

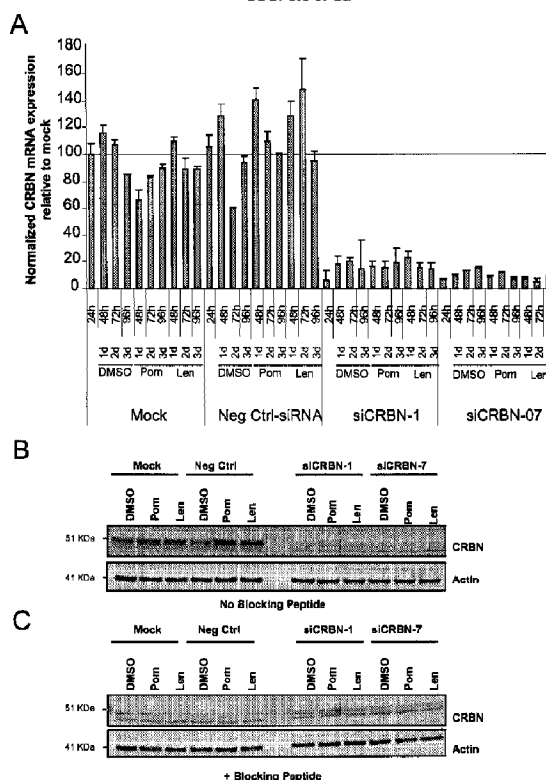


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CEREBLON EN TANT QUE PREDICTEUR
(54) Title: METHODS FOR THE TREATMENT OF CANCER AND INFLAMMATORY DISEASES USING CEREBLON AS
A PREDICTOR

FIG. 1A & 1B



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Uses of the protein cereblon as a predictor of clinical sensitivity to cancer, inflammatory diseases, and patient response to drug treatment.

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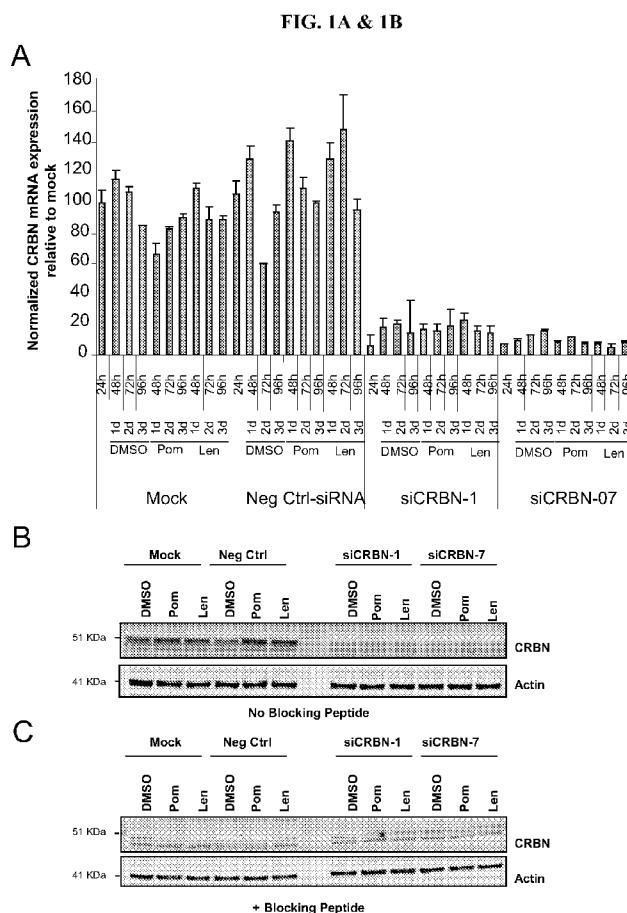
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METHODS FOR THE TREATMENT OF CANCER AND INFLAMMATORY DISEASES USING CEREBLON AS A PREDICTOR

The present application claims priority to U.S. Provisional Patent Application Nos. 61/481,066, filed April 29, 2011; 61/511,986, filed July 26, 2011; and 61/579,600, filed December 22, 2011; the entirety of each of which is incorporated herein by reference.

1. FIELD

Provided herein are uses of the protein cereblon as a predictor of clinical sensitivity to cancer and inflammatory diseases, and patient response to drug treatment.

2. BACKGROUND

2.1 Pathobiology of Cancer

Cancer is characterized primarily by an increase in the number of abnormal cells derived from a given normal tissue, invasion of adjacent tissues by these abnormal cells, or lymphatic or blood-borne spread of malignant cells to regional lymph nodes and to distant sites (metastasis). Clinical data and molecular biologic studies indicate that cancer is a multistep process that begins with minor preneoplastic changes, which may under certain conditions progress to neoplasia. The neoplastic lesion may evolve clonally and develop an increasing capacity for invasion, growth, metastasis, and heterogeneity, especially under conditions in which the neoplastic cells escape the host's immune surveillance. Roitt, I., Brostoff, J and Kale, D., *Immunology*, 17.1-17.12 (3rd ed., Mosby, St. Louis, Mo., 1993).

There is an enormous variety of cancers which are described in detail in the medical literature. Examples include cancers of the lung, colon, rectum, prostate, breast, brain, blood and intestine. The incidence of cancer continues to climb as the general population ages, as new cancers develop, and as susceptible populations (*e.g.*, people infected with AIDS or excessively exposed to sunlight) grow. However, options for the treatment of cancer are limited. For example, in the case of blood cancers (*e.g.*, multiple myeloma), few treatment options are available, especially when conventional chemotherapy fails and bone-marrow transplantation is not an option. A tremendous demand therefore exists for new methods and compositions that can be used to treat patients with cancer.

Many types of cancers are associated with new blood vessel formation, a process known as angiogenesis. Several of the mechanisms involved in tumor-induced angiogenesis have been elucidated. The most direct of these mechanisms is the secretion by the tumor cells of cytokines with angiogenic properties. Examples of these cytokines include acidic and basic fibroblastic growth factor (a,b-FGF), angiogenin, vascular endothelial growth factor (VEGF), and TNF- α . Alternatively, tumor cells can release angiogenic peptides through the production of proteases and the subsequent breakdown of the extracellular matrix where some cytokines are stored (*e.g.*, b-FGF). Angiogenesis can also be induced indirectly through the recruitment of inflammatory cells (particularly macrophages) and their subsequent release of angiogenic cytokines (*e.g.*, TNF- α , b-FGF).

Lymphoma refers to cancers that originate in the lymphatic system. Lymphoma is characterized by malignant neoplasms of lymphocytes—B lymphocytes and T lymphocytes (*i.e.*, B-cells and T-cells). Lymphoma generally starts in lymph nodes or collections of lymphatic tissue in organs including, but not limited to, the stomach or intestines. Lymphoma may involve the marrow and the blood in some cases. Lymphoma may spread from one site to other parts of the body.

The treatment of various forms of lymphomas are described, for example, in U.S. patent no. 7,468,363, the entirety of which is incorporated herein by reference. Such lymphomas include, but are not limited to, Hodgkin's lymphoma, non-Hodgkin's lymphoma, cutaneous B-cell lymphoma, activated B-cell lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), follicular center lymphoma, transformed lymphoma, lymphocytic lymphoma of intermediate differentiation, intermediate lymphocytic lymphoma (ILL), diffuse poorly differentiated lymphocytic lymphoma (PDL), centrocytic lymphoma, diffuse small-cleaved cell lymphoma (DSCCL), peripheral T-cell lymphomas (PTCL), cutaneous T-Cell lymphoma and mantle zone lymphoma and low grade follicular lymphoma.

Non-Hodgkin's lymphoma (NHL) is the fifth most common cancer for both men and women in the United States, with an estimated 63,190 new cases and 18,660 deaths in 2007. Jemal A, *et al.*, *CA Cancer J Clin* 2007; 57(1):43-66. The probability of developing NHL increases with age and the incidence of NHL in the elderly has been steadily increasing in the past decade, causing concern with the aging trend of the US population. *Id.* Clarke C A, *et al.*, *Cancer* 2002; 94(7):2015-2023.

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately one-third of non-Hodgkin's lymphomas. While some DLBCL patients are cured with traditional chemotherapy, the remainder die from the disease. Anticancer drugs cause rapid and persistent depletion of lymphocytes, possibly by direct apoptosis induction in mature T and B cells. See K. Stahnke, *et al.*, *Blood* 2001, 98:3066-3073. Absolute lymphocyte count (ALC) has been shown to be a prognostic factor in follicular non-Hodgkin's lymphoma and recent results have suggested that ALC at diagnosis is an important prognostic factor in diffuse large B-cell lymphoma.

The diffuse large-B-cell lymphomas (DLBCL) can be divided into distinct molecular subtypes according to their gene profiling patterns: germinal-center B-cell-like DLBCL (GCB-DLBCL), activated B-cell-like DLBCL (ABC-DLBCL), and primary mediastinal B-cell lymphoma (PMBL) or unclassified type. These subtypes are characterized by distinct differences in survival, chemo-responsiveness, and signaling pathway dependence, particularly the NF- κ B pathway. See D. Kim *et al.*, *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 8082. See Bea S, *et al.*, *Blood* 2005; 106: 3183-90; Ngo V.N. *et al.*, *Nature* 2011; 470: 115-9. Such differences have prompted the search for more effective and subtype-specific treatment strategies in DLBCL.

Leukemia refers to malignant neoplasms of the blood-forming tissues. Various forms of leukemias are described, for example, in U.S. patent no. 7,393,862 and U.S. provisional patent application no. 60/380,842, filed May 17, 2002, the entireties of which are incorporated herein by reference. Although viruses reportedly cause several forms of leukemia in animals, causes of leukemia in humans are to a large extent unknown. *The Merck Manual*, 944-952 (17th ed. 1999). Transformation to malignancy typically occurs in a single cell through two or more steps with subsequent proliferation and clonal expansion. In some leukemias, specific chromosomal translocations have been identified with consistent leukemic cell morphology and special clinical features (e.g., translocations of 9 and 22 in chronic myelocytic leukemia, and of 15 and 17 in acute promyelocytic leukemia). Acute leukemias are predominantly undifferentiated cell populations and chronic leukemias more mature cell forms.

Acute leukemias are divided into lymphoblastic (ALL) and non-lymphoblastic (ANLL) types. *The Merck Manual*, 946-949 (17th ed. 1999). They may be further subdivided by their morphologic and cytochemical appearance according to the French-American-British (FAB) classification or according to their type and degree of differentiation. The use of specific B- and

T-cell and myeloid-antigen monoclonal antibodies are most helpful for classification. ALL is predominantly a childhood disease which is established by laboratory findings and bone marrow examination. ANLL, also known as acute myelogenous leukemia or acute myeloid leukemia (AML), occurs at all ages and is the more common acute leukemia among adults; it is the form usually associated with irradiation as a causative agent.

Chronic leukemias are described as being lymphocytic (CLL) or myelocytic (CML). *The Merck Manual*, 949-952 (17th ed. 1999). CLL is characterized by the appearance of mature lymphocytes in blood, bone marrow, and lymphoid organs. The hallmark of CLL is sustained, absolute lymphocytosis ($> 5,000/\mu\text{L}$) and an increase of lymphocytes in the bone marrow. Most CLL patients also have clonal expansion of lymphocytes with B-cell characteristics. CLL is a disease of middle or old age. In CML, the characteristic feature is the predominance of granulocytic cells of all stages of differentiation in blood, bone marrow, liver, spleen, and other organs. In the symptomatic patient at diagnosis, the total white blood cell (WBC) count is usually about $200,000/\mu\text{L}$, but may reach $1,000,000/\mu\text{L}$. CML is relatively easy to diagnose because of the presence of the Philadelphia chromosome.

Bone marrow stromal cells are well known to support CLL disease progression and resistance to chemotherapy. Disrupting the interactions between CLL cells and stromal cells is an additional target of CLL chemotherapy.

In addition to the acute and chronic categorization, neoplasms are also categorized based upon the cells giving rise to such disorder into precursor or peripheral. *See e.g.*, U.S. patent publication no. 2008/0051379, the disclosure of which is incorporated herein by reference in its entirety. Precursor neoplasms include ALLs and lymphoblastic lymphomas and occur in lymphocytes before they have differentiated into either a T- or B-cell. Peripheral neoplasms are those that occur in lymphocytes that have differentiated into either T- or B-cells. Such peripheral neoplasms include, but are not limited to, B-cell CLL, B-cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma, follicular lymphoma, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, nodal marginal zone lymphoma, splenic marginal zone lymphoma, hairy cell leukemia, plasmacytoma, diffuse large B-cell lymphoma and Burkitt lymphoma. In over 95 percent of CLL cases, the clonal expansion is of a B cell lineage. *See Cancer: Principles & Practice of Oncology* (3rd Edition) (1989) (pp. 1843-1847). In less than 5 percent of CLL cases, the tumor cells have a T-cell phenotype.

Notwithstanding these classifications, however, the pathological impairment of normal hematopoiesis is the hallmark of all leukemias.

Multiple myeloma (MM) is a cancer of plasma cells in the bone marrow. Normally, plasma cells produce antibodies and play a key role in immune function. However, uncontrolled growth of these cells leads to bone pain and fractures, anemia, infections, and other complications.

Multiple myeloma is the second most common hematological malignancy, although the exact causes of multiple myeloma remain unknown. Multiple myeloma causes high levels of proteins in the blood, urine, and organs, including but not limited to M-protein and other immunoglobulins (antibodies), albumin, and beta-2-microglobulin. M-protein, short for monoclonal protein, also known as paraprotein, is a particularly abnormal protein produced by the myeloma plasma cells and can be found in the blood or urine of almost all patients with multiple myeloma.

Skeletal symptoms, including bone pain, are among the most clinically significant symptoms of multiple myeloma. Malignant plasma cells release osteoclast stimulating factors (including IL-1, IL-6 and TNF) which cause calcium to be leached from bones causing lytic lesions; hypercalcemia is another symptom. The osteoclast stimulating factors, also referred to as cytokines, may prevent apoptosis, or death of myeloma cells. Fifty percent of patients have radiologically detectable myeloma-related skeletal lesions at diagnosis. Other common clinical symptoms for multiple myeloma include polyneuropathy, anemia, hyperviscosity, infections, and renal insufficiency.

Bone marrow stromal cells are well known to support multiple myeloma disease progression and resistance to chemotherapy. Disrupting the interactions between multiple myeloma cells and stromal cells is an additional target of multiple myeloma chemotherapy.

Myelodysplastic syndrome (MDS) refers to a diverse group of hematopoietic stem cell disorders. MDS is characterized by a cellular marrow with impaired morphology and maturation (dysmyelopoiesis), peripheral blood cytopenias, and a variable risk of progression to acute leukemia, resulting from ineffective blood cell production. *See* The Merck Manual 953 (17th ed. 1999) and List et al., 1990, *J Clin. Oncol.* 8:1424. The treatment of MDS using immunomodulatory compounds is described in U.S. Patent Publication No. 2004/0220144, the entirety of which is hereby incorporated by reference.

Solid tumors are abnormal masses of tissue that may, but usually do not contain cysts or liquid areas. Solid tumors may be benign (not cancer), or malignant (cancer). Different types of solid tumors are named for the type of cells that form them. Examples of types solid tumors include, but are not limited to malignant melanoma, adrenal carcinoma, breast carcinoma, renal cell cancer, carcinoma of the pancreas, non-small-cell lung carcinoma (NSCLC) and carcinoma of unknown primary. Drugs commonly administered to patients with various types or stages of solid tumors include, but are not limited to, celebrex, etoposide, cyclophosphamide, docetaxel, apicitabine, IFN, tamoxifen, IL-2, GM-CSF, or a combination thereof.

While patients who achieve a complete remission after initial therapy have a good chance for cure, less than 10% of those who do not respond or relapse achieve a cure or a response lasting longer than 3 years. *See Cerny T, et al., Ann Oncol 2002; 13 Suppl 4:211-216.*

Rituximab is known to deplete normal host B cells. *See M. Aklilu et al., Annals of Oncology 15:1109-1114, 2004.* The long-term immunologic effects of B cell depletion with rituximab and the characteristics of the reconstituting B cell pool in lymphoma patients are not well defined, despite the widespread usage of this therapy. *See Jennifer H. Anolik et al., Clinical Immunology, vol. 122, issue 2, February 2007, pages 139-145.*

The approach for patients with relapsed or refractory disease relies heavily on experimental treatments followed by stem cell transplantation, which may not be appropriate for patients with a poor performance status or advanced age. Therefore, a tremendous demand exists for new methods that can be used to treat patients with NHL.

The link between cancer and altered cellular metabolism has been well established. *See Cairns, R.A., et al. Nature Rev., 2011, 11:85-95.* Understanding tumor cell metabolism and the associated genetic changes thereof may lead to the identification of improved methods of cancer treatment. *Id.* For example, tumor cell survival and proliferation via increased glucose metabolism has been linked to the PIK3 pathway, whereby mutations in tumor suppressor genes such as PTEN activate tumor cell metabolism. *Id.* AKT1 (*a.k.a.*, PKB) stimulates glucose metabolism associated with tumor cell growth by various interactions with PFKFB3, ENTPD5, mTOR and TSC2 (*a.k.a.*, tuberin). *Id.*

Transcription factors HIF1 and HIF2 are largely responsible for cellular response to low oxygen conditions often associated with tumors. *Id.* Once activated, HIF1 promotes tumor cell capacity to carry out glycolysis. *Id.* Thus, inhibition of HIF1 may slow or reverse tumor cell metabolism.

Activation of HIF1 has been linked to PI3K, tumor suppressor proteins such as VHL, succinate dehydrogenase (SDH) and fumarate hydratase. *Id.* The oncogenic transcription factor MYC has also been linked to tumor cell metabolism, specifically glycolysis. *Id.* MYC also promotes cell proliferation by glutamine metabolic pathways. *Id.*

- 5 AMP-activated protein kinase (AMPK) functions as a metabolic check point which tumor cells must overcome in order to proliferate. *Id.* Several mutations have been identified which suppress AMPK signaling in tumor cells. *See* Shackelford, D.B. & Shaw, R.J., *Nature Rev. Cancer*, 2009, 9: 563-575. STK11 has been identified as a tumor suppressor gene related to the role of AMPK. *See* Cairns, R.A., *et al. Nature Rev.*, 2011, 11:85-95.
- 10 The transcription factor p53, a tumor suppressor, also has an important role in the regulation of cellular metabolism. *Id.* The loss of p53 in tumor cells may be a significant contributor to changes in tumor cell metabolism to the glycolytic pathway. *Id.* The OCT1 transcription factor, another potential target for chemotherapeutics, may cooperate with p53 in regulating tumor cell metabolism. *Id.*
- 15 Pyruvate kinase M2 (PKM2) promotes changes in cellular metabolism which confer metabolic advantages to cancer cells by supporting cell proliferation. *Id.* For example, lung cancer cells which express PKM2 over PKM1 have been found to have such an advantage. *Id.* In the clinic, PKM2 has been identified as being overexpressed in a number of cancer types. *Id.* Thus PKM2 may be a useful biomarker for the early detection of tumors.
- 20 Mutations in isocitrate dehydrogenases IDH1 and IDH2 have been linked to tumorigenesis, specifically, in glioblastoma and acute myeloid leukemia. *See* Mardis, E.R. *et al.*, *N. Engl. J. Med.*, 2009, 361: 1058-1066; Parsons, D.W. *et al.*, *Science*, 2008, 321: 1807-1812.
- The incidence of cancer continues to climb as the general population ages, as new cancers develop, and as susceptible populations (*e.g.*, people infected with AIDS, the elderly or
- 25 excessively exposed to sunlight) grow. A tremendous demand therefore exists for new methods, treatments and compositions that can be used to treat patients with cancer including but not limited to those with lymphoma, NHL, multiple myeloma, AML, leukemias, and solid tumors.
- A variety of other diseases and disorders are also associated with, or characterized by, undesired angiogenesis. For example, enhanced or unregulated angiogenesis has been
- 30 implicated in a number of diseases and medical conditions including, but not limited to, ocular neovascular diseases, choroidal neovascular diseases, retina neovascular diseases, rubeosis

(neovascularization of the angle), viral diseases, genetic diseases, inflammatory diseases, allergic diseases, fibrosis, arthritis and autoimmune diseases. Examples of such diseases and conditions include, but are not limited to: diabetic retinopathy; retinopathy of prematurity; corneal graft rejection; neovascular glaucoma; retrolental fibroplasia; and proliferative vitreoretinopathy.

5 Accordingly, compounds that can control and/or inhibit unwanted angiogenesis or inhibit the production of certain cytokines, including TNF- α , may be useful in the treatment and prevention of various diseases and conditions.

2.2 Inflammatory Diseases

Inflammation plays a fundamental role in host defenses and the progression of immune-mediated diseases. The inflammatory response is initiated in response to injury (*e.g.*, trauma, ischemia, and foreign particles) and infection (*e.g.*, bacterial or viral infection) by a complex cascade of events, including chemical mediators (*e.g.*, cytokines and prostaglandins) and inflammatory cells (*e.g.*, leukocytes). The inflammatory response is characterized by increased blood flow, increased capillary permeability, and the influx of phagocytic cells. These events
10 result in swelling, redness, warmth (altered heat patterns), and pus formation at the site of injury or infection.

Cytokines and prostaglandins control the inflammatory response, and are released in an ordered and self-limiting cascade into the blood or affected tissues. This release of cytokines and prostaglandins increases the blood flow to the area of injury or infection, and may result in
20 redness and warmth. Some of these chemicals cause a leak of fluid into the tissues, resulting in swelling. This protective process may stimulate nerves and cause pain. These changes, when occurring for a limited period in the relevant area, work to the benefit of the body.

Tumor necrosis factor alpha (TNF- α) is a cytokine that is released primarily by mononuclear phagocytes in response to immunostimulators. TNF- α is capable of enhancing
25 most cellular processes, such as differentiation, recruitment, proliferation, and proteolytic degradation. At low levels, TNF- α confers protection against infective agents, tumors, and tissue damage. But TNF- α also has a role in many diseases. When administered to mammals or humans, TNF- α causes or aggravates inflammation, fever, cardiovascular effects, hemorrhage, coagulation, and acute phase responses similar to those seen during acute infections and shock
30 states. Enhanced or unregulated TNF- α production has been implicated in a number of diseases and medical conditions, for example, cancers, such as solid tumors and blood-borne tumors;

heart disease, such as congestive heart failure; and viral, genetic, inflammatory, allergic, and autoimmune diseases.

Adenosine 3',5'-cyclic monophosphate (cAMP) also plays a role in many diseases and conditions, such as but not limited to asthma and inflammation, and other conditions (Lowe and
5 Cheng, *Drugs of the Future*, 17(9), 799-807, 1992). It has been shown that the elevation of cAMP in inflammatory leukocytes inhibits their activation and the subsequent release of inflammatory mediators, including TNF- α and NF- κ B. Increased levels of cAMP also leads to the relaxation of airway smooth muscle.

A delicate well-balanced interplay between the humoral and cellular immune elements in
10 the inflammatory response enables the elimination of harmful agents and the initiation of the repair of damaged tissue. When this delicately balanced interplay is disrupted, the inflammatory response may result in considerable damage to normal tissue and may be more harmful than the original insult that initiated the reaction. In these cases of uncontrolled inflammatory responses, clinical intervention is needed to prevent tissue damage and organ dysfunction. Diseases such as
15 psoriasis, rheumatoid arthritis, osteoarthritis, psoriatic arthritis, Crohn's disease, asthma, allergies or inflammatory bowel disease, are characterized by chronic inflammation. Inflammatory diseases such as arthritis, related arthritic conditions (e.g., osteoarthritis, rheumatoid arthritis, and psoriatic arthritis), inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis), sepsis, psoriasis, atopic dermatitis, contact dermatitis, and chronic
20 obstructive pulmonary disease, chronic inflammatory pulmonary diseases are also prevalent and problematic ailments. Enhanced or unregulated TNF- α production plays a central role in the inflammatory response and the administration of their antagonists block chronic and acute responses in animal models of inflammatory disease.

Arthritis is a systemic autoimmune disease that can refer to a group of conditions
25 involving damage to the joints of the body. There are over 100 different forms of arthritis. The most common form is osteoarthritis (degenerative joint disease) and other arthritis forms are rheumatoid arthritis, psoriatic arthritis, and related autoimmune diseases such as lupus and gout. Rheumatoid arthritis is characterized by a chronic inflammation of the joints. Both synovial tissue and fluid are invaded by inflammatory cells which lead to cytokine production. T cells
30 and monocytes infiltrating the joints display an increased activation of Type 1 and 2 immune response markers.

Psoriatic arthritis is a chronic inflammatory arthritic condition affecting the skin, the joints, the insertion sites of tendons, ligaments, and fascia. Gladman, *Current Opinion in Rheumatology*, "Current concepts in psoriatic arthritis," 2002, 14:361-366, and Ruddy et al., *Rheumatology*, vol. 2., chapter 71, page 1071, 6th ed., 2001. Psoriatic arthritis is commonly associated with psoriasis. *Id.* Approximately 7% of patients with psoriasis develop psoriatic arthritis. The Merck Manual, 448 (17th ed., 1999). Psoriatic arthritis may appear in a variety of clinical patterns. There are five general patterns of psoriatic arthritis: arthritis of the distal interphalangeal joints, destructive arthritis, symmetric polyarthritis indistinguishable from rheumatoid arthritis, asymmetric oligoarthritis, and spondyloarthropathy. Ruddy et al., page 1073. Psoriasis appears to precede the onset of psoriatic arthritis in 60-80% of patients. Occasionally, arthritis and psoriasis appear simultaneously. Cutaneous eruptions may be preceded by the arthropathy.

Psoriasis is a chronic systemic autoimmune disease that appears on the skin. There are five types of psoriasis: plaque, guttate, inverse, pustular and erythrodermic. The most common form, plaque psoriasis, is commonly seen as red and white hues of scaly patches appearing on the top first layer of the epidermis. Some patients, though, have no dermatological symptoms. In plaque psoriasis, skin rapidly accumulates at these sites, which gives it a silvery-white appearance. Plaques frequently occur on the skin of the elbows and knees, but can affect any area, including the scalp, palms of hands and soles of feet, and genitals. In contrast to eczema, psoriasis is more likely to be found on the outer side of the joint. The disorder is a chronic recurring condition that varies in severity from minor localized patches to complete body coverage. Fingernails and toenails are frequently affected (psoriatic nail dystrophy) and can be seen as an isolated symptom. Psoriasis can also cause inflammation of the joints, which is known as psoriatic arthritis. In psoriasis, one hypothesis is that T cells become active, migrate to the dermis and trigger the release of cytokines, TNF- α in particular, which causes inflammation and the rapid proliferation of keratinocytes.

2.3 Compounds

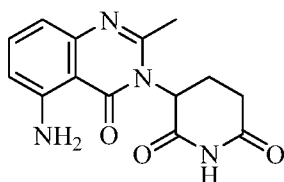
A number of studies have been conducted with the aim of providing compounds that can safely and effectively be used to treat diseases associated with abnormal production of TNF- α . See, e.g., Marriott, J.B., et al., *Expert Opin. Biol. Ther.*, 2001, 1(4): 1-8; G.W. Muller, et al., *J Med Chem.*, 1996, 39(17): 3238-3240; and G.W. Muller, et al., *Bioorg & Med Chem Lett.*, 1998, 8: 2669-

2674. Some studies have focused on a group of compounds selected for their capacity to potentially inhibit TNF- α production by LPS stimulated PBMC. L.G. Corral, *et al.*, *Ann. Rheum. Dis.*, 1999, 58:(Suppl I) 1107-1113. These compounds show not only potent inhibition of TNF- α but also marked inhibition of LPS induced monocyte IL1 β and IL12 production. LPS induced IL6 is also inhibited by such compounds, albeit partially. These compounds are potent stimulators of LPS induced IL10. *Id.*

Compounds for the methods provided herein include, but are not limited to, the substituted 2-(2,6-dioxopiperidin-3-yl) phthalimides and substituted 2-(2,6-dioxopiperidin-3-yl)-1-oxoisindoles described in U.S. patent nos. 6,281,230 and 6,316,471, both to G.W. Muller, *et al.* Still other specific compounds disclosed herein belong to a class of isoindole-imides disclosed in U.S. patent nos. 6,395,754, 6,555,554, 7,091,353, U.S. patent publication no. 2004/0029832, and International Publication No. WO 98/54170, each of which is incorporated herein by reference. Thalidomide, lenalidomide and pomalidomide have shown remarkable responses in patients with multiple myeloma, lymphoma and other hematological diseases such as myelodysplastic syndrome. *See Galustian C, et al., Expert Opin Pharmacother.*, 2009, 10:125-133. These drugs display a broad spectrum of activity, including anti-angiogenic properties, modulation of pro-inflammatory cytokines, co-stimulation of T cells, increased NK cell toxicity, direct anti-tumor effects and modulation of stem cell differentiation.

For example, thalidomide and lenalidomide have emerged as important options for the treatment of multiple myeloma in newly diagnosed patients, in patients with advanced disease who have failed chemotherapy or transplantation, and in patients with relapsed or refractory multiple myeloma. Lenalidomide in combination with dexamethasone has been approved for the treatment of patients with multiple myeloma who have received at least one prior therapy. Pomalidomide may also be administered in combination with dexamethasone. U.S. Patent Publication No. 2004/0029832 A1, the disclosure of which is hereby incorporated in its entirety, discloses the treatment of multiple myeloma.

Another compound provided herein is 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione ("Compound B"), which has the following structure:



or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

Compound B can be prepared according to the methods described in the Examples provided herein or as described in U.S. Pat. No. 7,635,700, the disclosure of which is
5 incorporated herein by reference in its entirety. The compound can be also synthesized according to other methods apparent to those of skill in the art based upon the teaching herein. In certain embodiments, Compound B is in a crystalline form described in U.S. Provisional Pat. App. No. 61/451,806, filed March 11, 2011, which is incorporated herein by reference in its
10 entirety. In some embodiments, the hydrochloride salt of Compound B is used in the methods provided herein. Methods of treating, preventing and/or managing cancers and other diseases using Compound B are described in U.S. Provisional Pat. App. No. 61/451,995, filed March 11, 2011, which is incorporated herein by reference in its entirety.

2.4 Cereblon

The protein Cereblon (CRBN) is a 442-amino acid protein conserved from plant to human. In
15 humans, the CRBN gene has been identified as a candidate gene of an autosomal recessive nonsyndromic mental retardation (ARNSMR). *See* Higgins, J.J. *et al.*, *Neurology*, 2004, 63:1927-1931. CRBN was initially characterized as an RGS-containing novel protein that interacted with a calcium-activated potassium channel protein (SLO1) in the rat brain, and was
20 later shown to interact with a voltage-gated chloride channel (CIC-2) in the retina with AMPK7 and DDB1. *See* Jo, S. *et al.*, *J. Neurochem*, 2005, 94:1212-1224; Hohberger B. *et al.*, *FEBS Lett*, 2009, 583:633-637; Angers S. *et al.*, *Nature*, 2006, 443:590-593. DDB1 was originally identified as a nucleotide excision repair protein that associates with damaged DNA binding
25 protein 2 (DDB2). Its defective activity causes the repair defect in the patients with xeroderma pigmentosum complementation group E (XPE). DDB1 also appears to function as a component of numerous distinct DCX (DDB1-CUL4-X-box) E3 ubiquitin-protein ligase complexes which
mediate the ubiquitination and subsequent proteasomal degradation of target proteins. CRBN has also been identified as a target for the development of therapeutic agents for diseases of the
cerebral cortex. *See* WO 2010/137547 A1.

Cereblon has recently been identified as a key molecular target that binds to thalidomide to cause
30 birth defects. *See* Ito, T. *et al.*, *Science*, 2010, 327:1345-1350. DDB1 was found to interact with CRBN and, thus, was indirectly associated with thalidomide. Moreover, thalidomide was

able to inhibit auto-ubiquitination of CRBN *in vitro*, suggesting that thalidomide is an E3 ubiquitin-ligase inhibitor. *Id.* Importantly, this activity was inhibited by thalidomide in wild-type cells, but not in cells with mutated CRBN binding sites that prevent thalidomide binding. *Id.* The thalidomide binding site was mapped to a highly conserved C-terminal 104 amino acid region in CRBN. *Id.* Individual point mutants in CRBN, Y384A and W386A were both defective for thalidomide binding, with the double point mutant having the lowest thalidomide-binding activity. *Id.* A link between CRBN and the teratogenic effect of thalidomide was confirmed in animal models of zebra-fish and chick embryos. *Id.*

Whether binding to CRBN, the CRBN E3 ubiquitin-ligase complex, or one or more substrates of CRBN, is required for the beneficial effects of thalidomide and other drugs is yet to be established. Understanding these interactions with thalidomide and other drug targets will allow the definition of the molecular mechanisms of efficacy and/or toxicity and may lead to drugs with improved efficacy and toxicity profiles.

2.5 Methods of Treating Cancer

Current cancer therapy may involve surgery, chemotherapy, hormonal therapy and/or radiation treatment to eradicate neoplastic cells in a patient (see, for example, Stockdale, 1998, *Medicine*, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). Recently, cancer therapy could also involve biological therapy or immunotherapy. All of these approaches may pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of a patient or may be unacceptable to the patient. Additionally, surgery may not completely remove neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue. Radiation therapy can also often elicit serious side effects. Hormonal therapy is rarely given as a single agent. Although hormonal therapy can be effective, it is often used to prevent or delay recurrence of cancer after other treatments have removed the majority of cancer cells. Certain biological and other therapies are limited in number and may produce side effects such as rashes or swellings, flu-like symptoms, including fever, chills and fatigue, digestive tract problems or allergic reactions.

With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of cancer. A number of cancer chemotherapeutics act by inhibiting DNA synthesis, either directly or indirectly by inhibiting the biosynthesis of deoxyribonucleotide triphosphate precursors, to prevent DNA replication and concomitant cell division. Gilman *et al.*, *Goodman*

and Gilman's: *The Pharmacological Basis of Therapeutics*, Tenth Ed. (McGraw Hill, New York).

Despite availability of a variety of chemotherapeutic agents, chemotherapy has many drawbacks. Stockdale, *Medicine*, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10, 1998.

5 Almost all chemotherapeutic agents are toxic, and chemotherapy causes significant and often dangerous side effects including severe nausea, bone marrow depression, and immunosuppression. Additionally, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents. In fact, those cells resistant to the particular chemotherapeutic agents
10 used in the treatment protocol often prove to be resistant to other drugs, even if those agents act by different mechanism from those of the drugs used in the specific treatment. This phenomenon is referred to as multidrug resistance. Because of the drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols.

Other diseases or conditions associated with, or characterized by, undesired angiogenesis
15 are also difficult to treat. However, some compounds such as protamine, heparin and steroids have been proposed to be useful in the treatment of certain specific diseases associated with, or characterized by, undesired angiogenesis. Taylor et al., *Nature* 297:307 (1982); Folkman et al., *Science* 221:719 (1983); and U.S. Pat. Nos. 5,001,116 and 4,994,443. Thalidomide and certain thalidomide derivatives based on their multiple activities have also been proposed for the
20 treatment of such diseases and conditions. U.S. Patent Nos. 5,593,990, 5,629,327, 5,712,291, 6,071,948 and 6,114,355 to D'Amato.

Still, there is a significant need for safe and effective methods of treating, preventing and managing cancer and other diseases that are refractory to standard treatments, such as surgery, radiation therapy, chemotherapy and hormonal therapy, while reducing or avoiding the toxicities
25 and/or side effects associated with the conventional therapies.

2.6 Methods of Treating Inflammatory Diseases

Current treatments for inflammatory diseases and disorders involve symptomatic medications and immunosuppressive agents to control symptoms. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, fenoprofen, naproxen, tolmetin,
30 sulindac, meclofenamate sodium, piroxicam, flurbiprofen, diclofenac, oxaprozin, nabumetone, etodolac, and ketoprofen have analgesic and anti-inflammatory effects. However, NSAIDs are

believed not to be capable of altering progression of the disease. (Tierney et al. (eds), *Current Medical Diagnosis & Treatment*, 37 ed., Appleton & Lange (1998), p 793). Moreover, NSAIDs frequently cause gastrointestinal side effects, affect the lower intestinal tract causing perforation or aggravating inflammatory bowel disease, produce renal toxicity, and prolong bleeding time.

5 Corticosteroids are another class of drugs that are commonly used to control inflammatory symptoms. Corticosteroids, like NSAIDs, do not alter the natural progression of the disease, and thus, clinical manifestations of active disease commonly reappear when the drug is discontinued. The serious problem of untoward reactions resulting from prolonged corticosteroid therapy (e.g., osteoporosis, increased risk of infection, increased appetite, hypertension, edema, peptic ulcers,
10 psychoses) greatly limits its long-term use.

Low doses of immunosuppressive agents such as cytotoxic agents may be used for the treatment of inflammatory disorders. For example, some treatments for psoriasis and arthritis are based on disease-modifying anti-rheumatic drugs (DMARDs such as cyclosporine A and methotrexate), anti-inflammatory agents (TNF- α inhibitors such as etanercept), and analgesics.

15 New treatments for inflammatory and autoimmune disorders are constantly being sought. In particular, any new treatment that reduces the dosage and/or frequency of administration of agents currently being used, or is capable of making a currently used treatment more effective is constantly being sought.

3. SUMMARY OF THE INVENTION

20 Provided herein are uses of the protein cereblon (CRBN) as a predictor of clinical sensitivity to cancer and inflammatory diseases, and patient response to treatment with the compounds provided herein. In certain embodiments, the compounds provided herein bind directly to CRBN-DDB1.

Also provided herein are methods for the treatment or management of cancer and
25 inflammatory diseases using CRBN as a predictive or prognostic factor for the compounds provided herein. In certain embodiments, provided herein are methods for screening or identifying cancer patients, e.g., multiple myeloma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, and/or MDS patients, for treatment with thalidomide, lenalidomide and/or pomalidomide, using CRBN levels as a
30 predictive or prognostic factor. In some embodiments, provided herein are methods for selecting

patients having a higher response rate to therapy with thalidomide, lenalidomide and/or pomalidomide, using CRBN levels as a predictive or prognostic factor.

In one embodiment, provided herein is a method of predicting patient response to treatment of cancer or an inflammatory disease with thalidomide, lenalidomide and/or pomalidomide, the method comprising obtaining biological material from the patient, and measuring the presence or absence of CRBN.

In one embodiment, the mRNA or protein is purified from the tumor and the presence or absence of a biomarker is measured by gene or protein expression analysis. In certain embodiments, the presence or absence of a biomarker is measured by quantitative real-time PCR (QRT-PCR), microarray, flow cytometry or immunofluorescence. In other embodiments, the presence or absence of a biomarker is measured by enzyme-linked immunosorbent assay-based methodologies (ELISA) or other similar methods known in the art. Biomarkers associated with non-Hodgkin's lymphomas are described, for example, in U.S. Patent Publication No. 2011/0223157, the entirety of which is incorporated by reference in its entirety.

In another embodiment, provided herein is a method of predicting patient response to treatment in a cancer patient, the method comprising obtaining cancer cells from the patient, culturing the cells in the presence or absence of a compound provided herein, purifying protein or RNA from the cultured cells, and measuring the presence or absence of a biomarker by, *e.g.*, protein or gene expression analysis. The expression monitored may be, for example, mRNA expression or protein expression. In one embodiment, the cancer patient is a lymphoma, leukemia, multiple myeloma, solid tumor, non-Hodgkin's lymphoma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma patient.

In another embodiment, provided herein is a method of monitoring tumor response to compound (*e.g.*, drug) treatment in a cancer patient. The method comprises obtaining a biological sample from the patient, measuring the expression of a biomarker in the biological sample, administering one or more compounds (*e.g.*, drugs) to the patient, thereafter obtaining a second biological sample from the patient, measuring biomarker expression in the second biological sample, and comparing the levels of expression, where an increased level of biomarker expression after treatment indicates the likelihood of an effective tumor response. In one embodiment, the cancer patient is a lymphoma, leukemia, multiple myeloma, solid tumor,

non-Hodgkin's lymphoma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma patient.

In one embodiment, a decreased level of biomarker expression after treatment indicates the likelihood of effective tumor response. The biomarker expression monitored can be, for example, mRNA expression or protein expression. The expression in the treated sample can increase, for example, by about 1.5X, 2.0X, 3X, 5X, or more. In one embodiment, the tumor is a lymphoma, leukemia, multiple myeloma, solid tumor, non-Hodgkin's lymphoma, DLBCL or melanoma.

In another embodiment, provided herein is a method of predicting the sensitivity to compound (*e.g.*, drug) treatment in a cancer patient, specifically, a multiple myeloma or non-Hodgkin's lymphoma patient (*e.g.*, DLBCL). The method comprises obtaining a biological sample from the patient, optionally isolating or purifying mRNA from the biological sample, amplifying the mRNA transcripts by, *e.g.*, RT-PCR, where a higher baseline level of a specific biomarker indicates a higher likelihood that the cancer will be sensitive to treatment with a compound (*e.g.*, drug). In certain embodiments, the biomarker is a gene or protein associated with multiple myeloma or non-Hodgkin's lymphoma (*e.g.*, DLBCL). In one embodiment, the genes are selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having lymphoma, leukemia, multiple myeloma, solid tumor, non-Hodgkin's lymphoma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, comprises identifying a gene or protein associated with CRBN. In one embodiment, the gene or protein associated with CRBN is selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having lymphoma, leukemia, multiple myeloma, a solid tumor, non-Hodgkin's lymphoma, DLBCL or melanoma sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione comprises measuring the level of CRBN activity in the patient. In another embodiment, measuring the level of CRBN activity in the patient comprises measuring

DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and/or NFκB in cells obtained from the patient.

In still other embodiments, provided herein are methods of predicting the sensitivity to compound (*e.g.*, drug) treatment in a patient having a disease or disorder selected from systemic
5 lupus erythematosus, ANCA-induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis and fibrotic conditions such as systemic sclerosis. The method comprises obtaining a biological sample from the patient, optionally isolating or purifying mRNA from the biological sample, amplifying the mRNA transcripts by, *e.g.*, RT-PCR, where a higher baseline level of a
10 specific biomarker indicates a higher likelihood that the disease or disorder will be sensitive to treatment with a compound (*e.g.*, drug). In certain embodiments, the biomarker is a gene or protein selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having systemic lupus erythematosus, ANCA-
15 induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis or systemic sclerosis and sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, comprises identifying a gene or protein associated with CRBN. In one embodiment, the gene or protein associated with CRBN is
20 selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having systemic lupus erythematosus, ANCA-
induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis or systemic sclerosis
25 sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione comprises measuring the level of CRBN activity in the patient. In another embodiment, measuring the level of CRBN activity in the patient comprises measuring DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and/or NFκB in cells obtained from the patient.

30 In one embodiment, the compound is thalidomide.

In another embodiment, the compound is lenalidomide.

In another embodiment, the compound is pomalidomide.

In another embodiment, the compound is 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, or an enantiomer thereof, or a pharmaceutically acceptable salt, polymorph, solvate or hydrate thereof.

5 Also provided herein are kits useful for predicting the likelihood of an effective lymphoma, leukemia, multiple myeloma, a solid tumor, non-Hodgkin's lymphoma, diffuse large B-cell lymphoma mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma treatment or for monitoring the effectiveness of a treatment with one or more compounds (*e.g.*, drugs). The kit comprises a solid support, and
10 a means for detecting the protein expression of at least one biomarker in a biological sample. Such a kit may employ, for example, a dipstick, a membrane, a chip, a disk, a test strip, a filter, a microsphere, a slide, a multiwell plate, or an optical fiber. The solid support of the kit can be, for example, a plastic, silicon, a metal, a resin, glass, a membrane, a particle, a precipitate, a gel, a polymer, a sheet, a sphere, a polysaccharide, a capillary, a film, a plate, or a slide. The biological
15 sample can be, for example, a cell culture, a cell line, a tissue, an oral tissue, gastrointestinal tissue, an organ, an organelle, a biological fluid, a blood sample, a urine sample, or a skin sample. The biological sample can be, for example, a lymph node biopsy, a bone marrow biopsy, or a sample of peripheral blood tumor cells.

 In another embodiment, the kit comprises a solid support, nucleic acids contacting the
20 support, where the nucleic acids are complementary to at least 20, 50, 100, 200, 350, or more bases of mRNA, and a means for detecting the expression of the mRNA in a biological sample.

 In certain embodiments, the kits provided herein employ means for detecting the expression of a biomarker by quantitative real-time PCR (QRT-PCR), microarray, flow
cytometry or immunofluorescence. In other embodiments, the expression of the biomarker is
25 measured by ELISA-based methodologies or other similar methods known in the art.

 In still other embodiments, the kits provided herein are useful for predicting the likelihood of an effective treatment of a disease or disorder selected from systemic lupus erythematosus, ANCA-induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis and
30 fibrotic conditions such as systemic sclerosis.

In addition to the methods described above, a compound provided herein is administered in combination with a therapy conventionally used to treat, prevent or manage a disease or disorder described herein. Examples of such conventional therapies include, but are not limited to, surgery, chemotherapy, radiation therapy, hormonal therapy, biological therapy and immunotherapy.

Also provided herein are pharmaceutical compositions, single unit dosage forms, dosing regimens and kits which comprise a compound provided herein, or a pharmaceutically acceptable salt, solvate, hydrate, stereoisomer, clathrate, or prodrug thereof, and a second, or additional, active agent. Second active agents include specific combinations, or “cocktails,” of drugs.

4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1A & 1B: Confirmation of CRBN knockdown by siRNAs in H929 and U266 multiple myeloma cells.

Figures 2A – 2C: Knockdown of CRBN abrogated G1 arrest induced by lenalidomide (“Len”), pomalidomide (“Pom”) and Compound B.

Figure 3A: CRBN knockdown in U266B1 cells confirmed by RT-PCR.

Figure 3B: CRBN knockdown abrogates lenalidomide and pomalidomide effect on cell cycle in U266 cells.

Figures 3C & 3D: Knockdown of CRBN prevents increase of p21^{WAF1} in lenalidomide and pomalidomide treated U266 cells as detected by RT-PCR and Western blot analysis.

Figures 4A – 4D: CRBN knockdown abrogates drug effect on phosphorylation of pRb and IRF-4 in H929 cells.

Figures 5A – 5C: Cell cycle and gene expression profiles in U266B1 cells transfected with CRBN-siRNA.

Figures 6A – 6D: DDB1 knockdown only partially affected cell cycle delay induced by lenalidomide and pomalidomide in U266 cells.

Figures 7A & 7B: Lenalidomide and pomalidomide decrease total K48-linked polyubiquitination but not K-63-linked ubiquitination in H929.

Figure 8: 1 hour up-regulated ubiquitination, no MG132.

Figure 9: 4 hour up-regulated ubiquitination, no MG132.

Figure 10: 1 hour up-regulated ubiquitination, MG132.

Figure 11: 4 hour up-regulated ubiquitination, MG132.

Figures 12A – 12D: Effect of CRBN knockdown on TNF α and IL-2 levels in T cells.

Figures 13A – 13C: Effect of CUL4A and CUL4A knockdown on TNF α and IL-2 levels
5 in T cells.

Figure 14: Antiproliferative activity of lenalidomide vs. CRBN expression in DLBCL
cells.

Figure 15A: Map of CRBN_034 clone.

Figure 15B: Map of DDB1_004 clone.

10 Figure 16A: Antiproliferative activity of lenalidomide in CRBN-sensitive myeloma cells.

Figure 16B: Antiproliferative activity of pomalidomide in CRBN-sensitive myeloma
cells.

Figure 16C: Antiproliferative activity of Compound B in CRBN-sensitive myeloma cells.

15 Figure 17: Peptides regulated by lenalidomide (“Len”) and pomalidomide (“Pom”) without MG132 in 1 hour ubiquitination experiments.

Figure 18: Peptides regulated by lenalidomide (“Len”) and pomalidomide (“Pom”) with
MG132 in 1 hour ubiquitination experiments.

Figure 19: Peptides regulated by lenalidomide (“Len”) and pomalidomide (“Pom”) without MG132 in 4 hour ubiquitination experiments.

20 Figure 20: Peptides regulated by lenalidomide (“Len”) and pomalidomide (“Pom”) with MG132 in 4 hour ubiquitination experiments.

Figure 21: Table of common peptides between lenalidomide (“Len”) and pomalidomide (“Pom”) in ubiquitination experiments.

25 Figure 22: Table of common hits in multiple lenalidomide (“Len”) and pomalidomide (“Pom”) ubiquitination experiments.

Figure 23: Ubiscan data for lenalidomide (“Len” or “Rev”), pomalidomide (“Pom”), and
Compound B.

Figure 24A: ABC-DLBCL signature genes.

Figure 24B: NF- κ B activity and IRF4 activity in DLBCL cells.

30 Figure 24C: “ABC Scores” of DLBCL cell lines.

Figure 25: Lenalidomide inhibits proliferation of ABC-DLBCL cells *in vitro*.

Figure 26: Lenalidomide treatment also induced apoptosis of sensitive cell lines such as OCI-Ly10.

Figures 27A - 27C: Effect of Lenalidomide on IRF4 expression in ABC-DLBCL cell lines.

5 Figure 28: Mutation analysis of CARD11 coiled-coil domain 1 in DLBCL cell lines.

Figures 29A – 29C: Lenalidomide inhibits activation of CARD11-Bcl-10-MALT1 complex in sensitive DLBCL cell lines.

Figures 30A - 30C: Lenalidomide inhibits NF- κ B activity in ABC-DLBCL cells.

10 Figures 31A – 31D: Alteration of IRF4 expression in ABC-DLBCL cells affects cell sensitivity to lenalidomide.

Figures 32A – 32D: Downregulation of IRF4, NF- κ B and proliferation by lenalidomide requires the presence of cereblon.

Figures 33A- 33B: Lenalidomide inhibits tumor growth in mouse xenograft model with OCI-Ly10 ABC-DLBCL.

15 Figures 34A – 34B: “ABC scores” and baseline IRF4/CRBN levels correlate lenalidomide sensitivity of DLBCL cells.

Figure 35: Alignment between heavy chain amino acid sequences of antibodies CGN-6-1-11 (top; SEQ ID NO:5) and CGN-6-4-5 (bottom; SEQ ID NO:8).

20 Figures 36: Alignment between light chain amino acid sequences of antibodies CGN-6-1-11 (top; SEQ ID NO:7) and CGN-6-4-5 (bottom; SEQ ID NO:11).

Figures 37A & 37B: Confocal immunofluorescent analysis of DF15 (left panel) and DF15R cells (right panel) using 1 μ g/ml CGN-6-4-5 antibody (green) (A) or CGN-6-4-5 antibody / CRBN blocking peptide mix (1:5 excess ratio) (B). Nuclear staining performed with Dapi (blue).

25 Figure 38: Immunoblot with myeloma cells containing endogenous CRBN (DF15), DF15R with no CRBN and HEK293 cells expressing recombinant flag-tagged CRBN.

Figure 39A & 39B: Binding of thalidomide and other compounds to CRBN

5. DETAILED DESCRIPTION OF THE INVENTION

30 The methods provided herein are based, in part, on the discovery that cereblon is associated with the anti-proliferative activities of certain drugs, such as the compounds provided

herein. In some embodiments, Cereblon (CRBN) may be utilized as a biomarker to indicate the effectiveness or progress of a disease treatment with a compound provided herein.

Without being bound to a particular theory, CRBN binding may contribute to or even be required for anti-proliferative or other activities of certain compounds, such as the compounds provided herein. In certain embodiments, the compounds provided herein bind directly to CRBN-DDB1 and/or the CRBN E3 ubiquitin-ligase complex. Mutations in CRBN could be associated with resistance to the compounds provided herein.

For example, the levels of CRBN were significantly lower in the pomalidomide-resistant cells line DF15R and the lenalidomide-resistant cells, H929 R10-1, H929 R10-2, H929 R10-3, H929 R10-4 and MM1/R compared to the matched parental lines. Furthermore, an interesting mutation was found in CRBN gene of one of the myeloma lines that had acquired resistance to lenalidomide while in the parental line the CRBN gene was wild type. This mutation mapped to the DDB1 binding domain in CRBN. Thus, in certain embodiments, the sensitivity of a cancer cell, *e.g.*, a myeloma cell, or a patient having cancer, to therapy with a compound provided herein is related to CRBN expression.

In relapsed or refractory diffuse large B-cell lymphoma (DLBCL), higher responses were seen in the activated B-cell-like (ABC) subtype than the germinal center B-cell-like subtype. As provided herein using DLBCL cell lines, it was shown that lenalidomide treatment preferentially suppressed proliferation of ABC-DLBCL cells *in vitro* and delayed tumor growth in a human tumor xenograft model, with minimal effect on non-ABC-DLBCL cells. This tumoricidal effect was associated with downregulation of interferon regulatory factor 4 (IRF4), a hallmark of ABC-DLBCL cells.

IRF4 inhibition by lenalidomide caused downregulation of B cell receptor (BCR)-dependent NF- κ B activation. While IRF4-specific siRNA mimicked effects of lenalidomide reducing NF- κ B activation, IRF4 overexpression enhanced NF- κ B activation and conferred resistance to lenalidomide. Furthermore, lenalidomide-induced IRF4 downregulation required the expression of CRBN. Without being bound to a particular theory, these data show that lenalidomide may have direct antitumor activity against DLBCL cells, preferentially ABC-DLBCL cells, by blocking IRF4 expression and the BCR-NF- κ B signaling pathway in a CRBN-dependent manner.

It has been proposed that CRBN protein functions as a substrate receptor for Cul4-E3-ligase complexes through its interaction with DDB1. As provided herein, whether *in vivo* ubiquitination is associated with drug responses in multiple myeloma cells has been investigated. In H929 cells, compounds provided herein decrease total K48-linked polyubiquitination but not K-63-linked ubiquitination after 30 minutes treatment. At present, nearly two dozen proteins are reported to be degraded by a Cul4-DDB1 ligase². Several studies have shown Cul4/DDB1-dependent ubiquitination of core histones, DNA repair proteins, cell cycle regulators and key signaling pathways molecules. mTORC1 signaling requires proteasomal function and the involvement of CUL4-DDB1 ubiquitin E3 ligase. Using CST Ubiscan technology, 162 unique ubiquitin-peptides were identified which were significantly modulated by the compounds provided herein after short treatments (1 – 4 h). The corresponding proteins participate in nucleosome and chromatin function, protein-DNA assembly and histone H2A. The relevance of this early modification in the mode of action of compounds provided herein, and the relationship with CRBN and CUL4/DDB1 activities are under investigation.

Provided herein are methods for the treatment or management of cancer and inflammatory diseases using CRBN as a predictive or prognostic factor for the compounds provided herein. In certain embodiments, provided herein are methods for screening or identifying cancer patients, *e.g.*, lymphoma, leukemia, multiple myeloma, solid tumor, non-Hodgkin's lymphoma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma patients, for treatment with thalidomide, lenalidomide and/or pomalidomide, using CRBN levels as a predictive or prognostic factor. In some embodiments, provided herein are methods for selecting patients having a higher response rate to therapy with thalidomide, lenalidomide and/or pomalidomide, using CRBN levels as a predictive or prognostic factor.

In one embodiment, provided herein is a method of predicting patient response to treatment of cancer or an inflammatory disease with thalidomide, lenalidomide and/or pomalidomide, the method comprising obtaining biological material from the patient, and measuring the presence or absence of CRBN.

In one embodiment, the mRNA or protein is purified from the tumor and the presence or absence of a biomarker is measured by gene or protein expression analysis. In certain embodiments, the presence or absence of a biomarker is measured by quantitative real-time PCR

(QRT-PCR), microarray, flow cytometry or immunofluorescence. In other embodiments, the presence or absence of a biomarker is measured by enzyme-linked immunosorbent assay-based methodologies (ELISA) or other similar methods known in the art.

In another embodiment, provided herein is a method of predicting the sensitivity to
5 compound (*e.g.*, drug) treatment in a cancer patient, such as, a multiple myeloma or non-Hodgkin's lymphoma patient. The method comprises obtaining a biological sample from the patient, optionally isolating or purifying mRNA from the biological sample, amplifying the mRNA transcripts by, *e.g.*, RT-PCR, where a higher baseline level of a specific biomarker indicates a higher likelihood that the cancer will be sensitive to treatment with a compound (*e.g.*,
10 drug). In certain embodiments, the biomarker is a gene or protein associated with multiple myeloma or non-Hodgkin's lymphoma (*e.g.*, DLBCL). In one embodiment, the genes are selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having lymphoma, leukemia, multiple myeloma,
15 a solid tumor, non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, comprises identifying a gene or protein associated with CRBN. In one embodiment, the gene or protein associated with
20 CRBN is selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having lymphoma, leukemia, multiple myeloma, solid tumor, non-Hodgkin's lymphoma, DLBCL, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, MDS or melanoma sensitive to
25 treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione comprises measuring the level of CRBN activity in the patient. In another embodiment, measuring the level of CRBN activity in the patient comprises measuring DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and/or NFκB in cells obtained from the patient.

30 In one embodiment, the compound is thalidomide.

In another embodiment, the compound is lenalidomide.

In another embodiment, the compound is pomalidomide.

In another embodiment, the compound is 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, or an enantiomer thereof, or a pharmaceutically acceptable salt, polymorph, solvate or hydrate thereof.

5 In one embodiment, the cancer is multiple myeloma.

In another embodiment, the cancer is non-Hodgkin's lymphoma. In one embodiment, the non-Hodgkin's lymphoma is of the activated B-cell phenotype.

In another embodiment, the cancer is diffuse large B-cell lymphoma. In one embodiment, the diffuse large B-cell lymphoma is of the activated B-cell phenotype.

10 In another embodiment, the cancer is mantle cell lymphoma.

In another embodiment, the cancer is follicular lymphoma.

In another embodiment, the cancer is acute myeloblastic leukemia.

In another embodiment, the cancer is chronic lymphocytic leukemia.

In another embodiment, the cancer is myelodysplastic syndrome.

15 In another embodiment, the cancer is melanoma.

In still other embodiments, provided herein are methods of predicting the sensitivity to compound (*e.g.*, drug) treatment in a patient having a disease or disorder selected from systemic lupus erythematosus, ANCA-induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis and fibrotic conditions such as systemic sclerosis. The method comprises obtaining a biological sample from the patient, optionally isolating or purifying mRNA from the biological sample, amplifying the mRNA transcripts by, *e.g.*, RT-PCR, where a higher baseline level of a specific biomarker indicates a higher likelihood that the disease or disorder will be sensitive to treatment with a compound (*e.g.*, drug). In certain embodiments, the biomarker is a gene or protein selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

25 In one embodiment, identifying a patient having selected from systemic lupus erythematosus, ANCA-induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis or systemic sclerosis sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione comprises identification of a

gene or protein associated with CRBN. In one embodiment, the gene or protein associated with CRBN is selected from the group consisting of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and NFκB.

In one embodiment, identifying a patient having systemic lupus erythematosus, ANCA-
5 induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis or systemic sclerosis sensitive to treatment with thalidomide, lenalidomide, pomalidomide and/or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione comprises measuring the level of CRBN activity in the patient. In another embodiment, measuring the level of CRBN activity in the
10 patient comprises measuring DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 and/or NFκB in cells obtained from the patient.

In one embodiment, the compound is thalidomide.

In another embodiment, the compound is lenalidomide.

In another embodiment, the compound is pomalidomide.

15 In another embodiment, the compound is 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, or an enantiomer thereof, or a pharmaceutically acceptable salt, polymorph, solvate or hydrate thereof.

Also provided herein are kits useful for predicting the likelihood of an effective lymphoma, leukemia, multiple myeloma, a solid tumor, non-Hodgkin's lymphoma, diffuse large
20 B-cell lymphoma, mantle cell lymphoma, follicular lymphoma, acute myeloblastic leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome or melanoma treatment or for monitoring the effectiveness of a treatment with one or more compounds (*e.g.*, drugs). The kit comprises a solid support, and a means for detecting the protein expression of at least one biomarker in a biological sample. Such a kit may employ, for example, a dipstick, a membrane,
25 a chip, a disk, a test strip, a filter, a microsphere, a slide, a multiwell plate, or an optical fiber. The solid support of the kit can be, for example, a plastic, silicon, a metal, a resin, glass, a membrane, a particle, a precipitate, a gel, a polymer, a sheet, a sphere, a polysaccharide, a capillary, a film, a plate, or a slide. The biological sample can be, for example, a cell culture, a cell line, a tissue, an oral tissue, gastrointestinal tissue, an organ, an organelle, a biological fluid,
30 a blood sample, a urine sample, or a skin sample. The biological sample can be, for example, a lymph node biopsy, a bone marrow biopsy, or a sample of peripheral blood tumor cells.

In another embodiment, the kit comprises a solid support, nucleic acids contacting the support, where the nucleic acids are complementary to at least 20, 50, 100, 200, 350, or more bases of mRNA, and a means for detecting the expression of the mRNA in a biological sample.

In certain embodiments, the kits provided herein employ means for detecting the expression of a biomarker by quantitative real-time PCR (QRT-PCR), microarray, flow cytometry or immunofluorescence. In other embodiments, the expression of the biomarker is measured by ELISA-based methodologies or other similar methods known in the art.

In still other embodiments, the kits provided herein are useful for predicting the likelihood of an effective treatment of a disease or disorder selected from systemic lupus erythematosus, ANCA-induced vasculitis, glomerulonephritis, acute Wegener's granulomatosis, Myasthenia Gravis, Sjogren Syndrome, anti-phospholipid syndrome, rheumatoid arthritis and fibrotic conditions such as systemic sclerosis.

Provided herein is a method of selecting a group of cancer patients based on the level of CRBN expression, or the levels of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 or NFκB expression within the cancer, for the purposes of predicting clinical response, monitoring clinical response, or monitoring patient compliance to dosing by thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof; wherein the cancer patients are selected from multiple myeloma, non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, melanoma and solid tumor patients.

In one embodiment, the cancer patients are multiple myeloma patients.

In one embodiment, cancer patients are non-Hodgkin's lymphoma patients. In one embodiment, the non-Hodgkin's lymphoma is of the activated B-cell phenotype.

In one embodiment, cancer patients are diffuse large B-cell lymphoma patients. In one embodiment, the diffuse large B-cell lymphoma is of the activated B-cell phenotype.

In one embodiment, method of selecting a group of cancer patients is based on the level of DDB1 expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of DDB2 expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of GSK3B expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of CUL4A expression within the cancer.

5 In one embodiment, the method of selecting a group of cancer patients is based on the level of CUL4B expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of XBP-1 expression within the cancer.

10 In one embodiment, the method of selecting a group of cancer patients is based on the level of FAS1 expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of RANBP6 expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of DUS3L expression within the cancer.

15 In one embodiment, the method of selecting a group of cancer patients is based on the level of PHGDH expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of AMPK expression within the cancer.

20 In one embodiment, the method of selecting a group of cancer patients is based on the level of IRF4 expression within the cancer.

In one embodiment, the method of selecting a group of cancer patients is based on the level of NFκB expression within the cancer.

25 Also provided herein is a method of identifying or monitoring multiple myeloma patient resistance to thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione therapy, based on the presence or appearance of mutations within a CRBN gene.

In one embodiment, the mutation with the CRBN gene is a single-nucleotide polymorphism in the coding region c.745C>CA causing an amino acid change 249D>YD in the protein within the DDB1 binding domain of CRBN.

30 In another embodiment, provided herein is a method of selecting a group of patients responsive to treatment with thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-

4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof; based on the level of CRBN expression, or the levels of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 or NFκB expression within the patient's T cells, B cells, or plasma cells, for the purposes of predicting clinical response, monitoring clinical response, or monitoring patient compliance to dosing by thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

Also provided herein is an isolated CRBN antibody, for example, "CRBN70," as prepared according to Example 6.20 or 6.21 below. In one embodiment, the antibody is a polyclonal antibody. In another embodiment, the antibody is a monoclonal antibody. In some embodiments, the antibody is a rabbit polyclonal antibody. In other embodiments, the antibody is a rabbit monoclonal antibody.

In another embodiment, provided herein is an isolated antibody which immunospecifically binds to the epitope having an amino acid sequence EEFHGRTLHDDDC (SEQ ID:1). In another embodiment, the antibody immunospecifically binds to the epitope having an amino acid sequence EEFHGRTLHDDDC (SEQ ID:1), wherein the peptide is coupled to Keyhole Limpet Hemocyanin (KLH). In one embodiment, the antibody is a polyclonal antibody. In another embodiment, the antibody is a monoclonal antibody. In some embodiments, the antibody is a rabbit polyclonal antibody. In other embodiments, the antibody is a rabbit monoclonal antibody. In certain embodiments, the antibody immunospecifically binds peptide 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12).

In certain embodiments, provided herein is an antibody that immunospecifically binds CRBN and comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:5. In other embodiment, the antibody immunospecifically binds CRBN and comprises a light chain having the amino acid sequence depicted in SEQ ID NO:7. In some embodiments, the antibody comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:5 and a light chain having the amino acid sequence depicted in SEQ ID NO:7. In certain embodiments, the antibody immunospecifically binds CRBN and comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:9. In other embodiment, the antibody immunospecifically

binds CRBN and comprises a light chain having the amino acid sequence depicted in SEQ ID NO:11. In some embodiments, the antibody comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:9 and a light chain having the amino acid sequence depicted in SEQ ID NO:11. In certain embodiments, the antibody immunospecifically binds peptide 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12).

Also provided herein is a method of utilizing a CRBN antibody (*e.g.*, a rabbit polyclonal or monoclonal antibody CRBN70, or a rabbit polyclonal or monoclonal antibody that binds peptide 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12) to measure expression levels of CRBN in patient tumor or host cells, to predict clinical response, monitor clinical response, monitor patient compliance to dosing, or monitor development of resistance to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof. In one embodiment, the CRBN antibody immunospecifically binds to the epitope having an amino acid sequence EEFHGRTLHDDD (SEQ ID NO:1). In an embodiment, the CRBN antibody specifically binds to EEFHGRTLHDDD (SEQ ID NO:1), which is coupled to Keyhole Limpet Hemocyanin (KLH). In one embodiment, the CRBN antibody is a polyclonal antibody, such as a rabbit polyclonal antibody. In another embodiment, the CRBN antibody is a monoclonal antibody, such as a rabbit monoclonal antibody.

5.1 Definitions

As used herein, and unless otherwise specified, the terms “treat,” “treating” and “treatment” refer to an action that occurs while a patient is suffering from the specified cancer, which reduces the severity of the cancer, or retards or slows the progression of the cancer.

The term “sensitivity” and “sensitive” when made in reference to treatment with compound is a relative term which refers to the degree of effectiveness of the compound in lessening or decreasing the progress of a tumor or the disease being treated. For example, the term “increased sensitivity” when used in reference to treatment of a cell or tumor in connection with a compound refers to an increase of, at least a 5%, or more, in the effectiveness of the tumor treatment.

As used herein, and unless otherwise specified, the term “therapeutically effective amount” of a compound is an amount sufficient to provide a therapeutic benefit in the treatment

or management of a cancer, or to delay or minimize one or more symptoms associated with the presence of the cancer. A therapeutically effective amount of a compound means an amount of therapeutic agent, alone or in combination with other therapies, which provides a therapeutic benefit in the treatment or management of the cancer. The term “therapeutically effective amount” can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of cancer, or enhances the therapeutic efficacy of another therapeutic agent.

As used herein, an “effective patient tumor response” refers to any increase in the therapeutic benefit to the patient. An “effective patient tumor response” can be, for example, a 5%, 10%, 25%, 50%, or 100% decrease in the rate of progress of the tumor. An “effective patient tumor response” can be, for example, a 5%, 10%, 25%, 50%, or 100% decrease in the physical symptoms of a cancer. An “effective patient tumor response” can also be, for example, a 5%, 10%, 25%, 50%, 100%, 200%, or more increase in the response of the patient, as measured by any suitable means, such as gene expression, cell counts, assay results, etc.

The term “likelihood” generally refers to an increase in the probability of an event. The term “likelihood” when used in reference to the effectiveness of a patient tumor response generally contemplates an increased probability that the rate of tumor progress or tumor cell growth will decrease. The term “likelihood” when used in reference to the effectiveness of a patient tumor response can also generally mean the increase of indicators, such as mRNA or protein expression, that may evidence an increase in the progress in treating the tumor.

The term “predict” generally means to determine or tell in advance. When used to “predict” the effectiveness of a cancer treatment, for example, the term “predict” can mean that the likelihood of the outcome of the cancer treatment can be determined at the outset, before the treatment has begun, or before the treatment period has progressed substantially.

The term “monitor,” as used herein, generally refers to the overseeing, supervision, regulation, watching, tracking, or surveillance of an activity. For example, the term “monitoring the effectiveness of a compound” refers to tracking the effectiveness in treating a cancer in a patient or in a tumor cell culture. Similarly, the “monitoring,” when used in connection with patient compliance, either individually, or in a clinical trial, refers to the tracking or confirming that the patient is actually taking a drug being tested as prescribed. The monitoring can be performed, for example, by following the expression of mRNA or protein biomarkers.

An improvement in the cancer or cancer-related disease can be characterized as a complete or partial response. "Complete response" refers to an absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid (CSF) or abnormal monoclonal protein measurements. "Partial response" refers to at least about a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% decrease in all measurable tumor burden (*i.e.*, the number of malignant cells present in the subject, or the measured bulk of tumor masses or the quantity of abnormal monoclonal protein) in the absence of new lesions. The term "treatment" contemplates both a complete and a partial response.

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

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, blood-borne tumors (*e.g.*, multiple myeloma, lymphoma and leukemia), and solid tumors.

The term "refractory or resistant" refers to a circumstance where patients, even after intensive treatment, have residual cancer cells (*e.g.*, leukemia or lymphoma cells) in their lymphatic system, blood and/or blood forming tissues (*e.g.*, marrow).

As used herein the terms "polypeptide" and "protein" as used interchangeably herein, refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term polypeptide as used herein can also refer to a peptide. The amino acids making up the polypeptide may be naturally derived, or may be synthetic. The polypeptide can be purified from a biological sample.

The term "antibody" is used herein in the broadest sense and covers fully assembled antibodies, antibody fragments which retain the ability to specifically bind to the antigen (*e.g.*, Fab, F(ab')₂, Fv, and other fragments), single chain antibodies, diabodies, antibody chimeras, hybrid antibodies, bispecific antibodies, humanized antibodies, and the like. The term "antibody" covers both polyclonal and monoclonal antibodies.

The term “antibody” and “immunoglobulin” or “Ig” may be used interchangeably herein. The terms “antibodies that immunospecifically bind to a CRBN antigen,” “antibodies that immunospecifically bind to a CRBN epitope,” “CRBN antibodies,” “anti-CRBN antibodies” and analogous terms are also used interchangeably herein and refer to antibodies and fragments thereof, that specifically bind to a CRBN polypeptide, such as a CRBN antigen or epitope (*e.g.*, EEFHGRTLHDDD (SEQ ID NO:1) or peptide 65-76 human CRBN (SEQ ID NO:12)). The antibodies, including both modified antibodies (*i.e.*, antibodies that comprise a modified IgG (*e.g.*, IgG1) constant domain and unmodified antibodies (*i.e.*, antibodies that do not comprise a modified IgG (*e.g.*, IgG1) constant domain that specifically bind to a CRBN polypeptide. An antibody or a fragment thereof that immunospecifically binds to a CRBN antigen may be cross-reactive with related antigens. In certain embodiments, an antibody or a fragment thereof that immunospecifically binds to a CRBN antigen does not cross-react with other antigens. An antibody or a fragment thereof that immunospecifically binds to a CRBN antigen can be identified, for example, by immunoassays, BIAcore, or other techniques known to those of skill in the art. An antibody or a fragment thereof binds specifically to a CRBN antigen when it binds to a CRBN antigen with higher affinity than to any cross-reactive antigen as determined using experimental techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISAs). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 times background. *See, e.g.*, Paul, ed., 1989, Fundamental Immunology Second Edition, Raven Press, New York at pages 332-336 for a discussion regarding antibody specificity.

Antibodies provided herein include, but are not limited to, synthetic antibodies, monoclonal antibodies, recombinantly produced antibodies, multispecific antibodies (including bi-specific antibodies), human antibodies, humanized antibodies, chimeric antibodies, intrabodies, single-chain Fvs (scFv) (*e.g.*, including monospecific, bispecific, etc.), camelized antibodies, Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv), anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. In particular, antibodies provided herein include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, antigen binding domains or molecules that contain an antigen-binding site that immunospecifically binds to a CRBN antigen (*e.g.*, one or more complementarity determining regions (CDRs) of an anti-CRBN antibody). The antibodies

provided herein can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), any class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or any subclass (*e.g.*, IgG2a and IgG2b) of immunoglobulin molecule. In some embodiments, the anti-CRBN antibodies are fully human, such as fully human monoclonal CRBN antibodies. In certain embodiments, antibodies provided herein are
5 IgG antibodies, or a class (*e.g.*, human IgG1 or IgG4) or subclass thereof.

The term “antigen binding domain,” “antigen binding region,” “antigen binding fragment,” and similar terms refer to that portion of an antibody which comprises the amino acid residues that interact with an antigen and confer on the binding agent its specificity and affinity for the antigen (*e.g.*, the CDR). The antigen binding region can be derived from any animal
10 species, such as rodents (*e.g.*, rabbit, rat or hamster) and humans. In some embodiments, the antigen binding region will be of human origin.

The term “constant region” or “constant domain” of an antibody refers to a carboxy terminal portion of the light and heavy chain which is not directly involved in binding of the antibody to antigen but exhibits various effector function, such as interaction with the Fc
15 receptor. The terms refer to the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable domain, which contains the antigen binding site. The constant domain contains the CH1, CH2 and CH3 domains of the heavy chain and the CL domain of the light chain.

The term “epitope” as used herein refers to a localized region on the surface of an antigen,
20 such as CRBN polypeptide or CRBN polypeptide fragment, that is capable of being bound to one or more antigen binding regions of an antibody, and that has antigenic or immunogenic activity in an animal, such as a mammal (*e.g.*, a human), that is capable of eliciting an immune response. An epitope having immunogenic activity is a portion of a polypeptide that elicits a antibody response in an animal. An epitope having antigenic activity is a portion of a polypeptide to
25 which an antibody immunospecifically binds as determined by any method well known in the art, for example, by the immunoassays described herein. Antigenic epitopes need not necessarily be immunogenic. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. A region of a polypeptide contributing
30 to an epitope may be contiguous amino acids of the polypeptide or the epitope may come together from two or more non-contiguous regions of the polypeptide. The epitope may or may

not be a three-dimensional surface feature of the antigen. An exemplary epitope of CRBN provided herein is EEFHGRTLHDDD (SEQ ID NO:1) or peptide 65-60 of CRBN (SEQ ID NO:13).

The terms “fully human antibody” or “human antibody” are used interchangeably herein and refer to an antibody that comprises a human variable region and, in some embodiments, a human constant region. In specific embodiments, the terms refer to an antibody that comprises a variable region and constant region of human origin. “Fully human” anti-CRBN antibodies, in certain embodiments, can also encompass antibodies which bind CRBN polypeptides and are encoded by nucleic acid sequences which are naturally occurring somatic variants of human germline immunoglobulin nucleic acid sequence. In a specific embodiment, the anti-CRBN antibodies provided herein are fully human antibodies. The term “fully human antibody” includes antibodies having variable and constant regions corresponding to human germline immunoglobulin sequences as described by Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, 1991. Exemplary methods of producing fully human antibodies are provided, *e.g.*, in the Examples herein, but any method known in the art may be used.

The phrase “recombinant human antibody” includes human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library, antibodies isolated from an animal (*e.g.*, a mouse or cow) that is transgenic and/or transchromosomal for human immunoglobulin genes (see, *e.g.*, Taylor, L. D. et al. (1992) Nucl. Acids Res. 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies can have variable and constant regions derived from human germline immunoglobulin sequences. See Kabat, E. A. *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant

antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term “heavy chain” when used in reference to an antibody refers to five distinct types, called alpha (α), delta (δ), epsilon (ϵ), gamma (γ) and mu (μ), based on the amino acid sequence of the heavy chain constant domain. These distinct types of heavy chains are well known and give rise to five classes of antibodies, IgA, IgD, IgE, IgG and IgM, respectively, including four subclasses of IgG, namely IgG1, IgG1, IgG3 and IgG4. In some embodiments the heavy chain is a human heavy chain.

The terms “Kabat numbering,” and like terms are recognized in the art and refer to a system of numbering amino acid residues which are more variable (i.e. hypervariable) than other amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen binding portion thereof. Kabat *et al.* (1971) *Ann. any Acad. Sci.* 190:382-391 and, Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242. For the heavy chain variable region, the hypervariable region typically ranges from amino acid positions 31 to 35 for CDR1, amino acid positions 50 to 65 for CDR2, and amino acid positions 95 to 102 for CDR3. For the light chain variable region, the hypervariable region typically ranges from amino acid positions 24 to 34 for CDR1, amino acid positions 50 to 56 for CDR2, and amino acid positions 89 to 97 for CDR3. Other numbering schemes will be readily understood by those skilled in the art.

The term “light chain” when used in reference to an antibody refers to two distinct types, called kappa (κ) or lambda (λ) based on the amino acid sequence of the constant domains. Light chain amino acid sequences are well known in the art. In certain embodiments, the light chain is a human light chain.

The term “monoclonal antibody” refers to an antibody obtained from a population of homogenous or substantially homogeneous antibodies, and each monoclonal antibody will typically recognize a single epitope on the antigen. In some embodiments, a “monoclonal antibody,” as used herein, is an antibody produced by a single hybridoma or other cell, wherein the antibody immunospecifically binds to only a CRBN epitope as determined, e.g., by ELISA or other antigen-binding or competitive binding assay known in the art or in the Examples provided herein. The term “monoclonal” is not limited to any particular method for making the antibody. For example, monoclonal antibodies provided herein may be made by the hybridoma method as

described in Kohler et al.; Nature, 256:495 (1975) or may be isolated from phage libraries using the techniques as described herein, for example. Other methods for the preparation of clonal cell lines and of monoclonal antibodies expressed thereby are well known in the art. *See, e.g.*, Chapter 11 in: Short Protocols in Molecular Biology, (2002) 5th Ed., Ausubel et al., eds., John Wiley and Sons, New York. Other exemplary methods of producing other monoclonal antibodies are provided in the Examples herein.

“Polyclonal antibodies” as used herein refers to an antibody population generated in an immunogenic response to a protein having many epitopes and thus includes a variety of different antibodies directed to the same and to different epitopes within the protein. Methods for producing polyclonal antibodies are known in the art. *See, e.g.*, Chapter 11 in: Short Protocols in Molecular Biology, (2002) 5th Ed., Ausubel et al., eds., John Wiley and Sons, New York.

The terms “cereblon” or “CRBN” and similar terms refers to the polypeptides (“polypeptides,” “peptides” and “proteins” are used interchangeably herein) comprising the amino acid sequence any CRBN, such as a human CRBN protein (*e.g.*, human CRBN isoform 1, GenBank Accession No. NP_057386 (SEQ ID NO:12); or human CRBN isoforms 2, GenBank Accession No. NP_001166953 (SEQ ID NO:13), each of which is herein incorporated by reference in its entirety), and related polypeptides, including SNP variants thereof. Related CRBN polypeptides include allelic variants (*e.g.*, SNP variants); splice variants; fragments; derivatives; substitution, deletion, and insertion variants; fusion polypeptides; and interspecies homologs, which, in certain embodiments, retain CRBN activity and/or are sufficient to generate an anti-CRBN immune response.

The term “CRBN antigen” refers to that portion of a CRBN polypeptide to which an antibody immunospecifically binds. A CRBN antigen also refers to an analog or derivative of a CRBN polypeptide or fragment thereof to which an antibody immunospecifically binds. A localized region on the surface of a CRBN antigen that is capable of eliciting an immune response is an CRBN “epitope.” A region of a CRBN polypeptide contributing to an epitope may be contiguous amino acids of the polypeptide or the epitope may come together from two or more non-contiguous regions of the polypeptide. The epitope may or may not be a three-dimensional surface feature of the antigen. In certain embodiments, the CRBN epitope is EEFHGRTLHDDD (SEQ ID NO:1) or peptide 65-76 of human CRBN (SEQ ID NO:12).

The term “variable region” or “variable domain” refers to a portion of the light and heavy chains, typically about the amino-terminal 120 to 130 amino acids in the heavy chain and about 100 to 110 amino acids in the light chain, which differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen.

5 The variability in sequence is concentrated in those regions called complementarily determining regions (CDRs) while the more highly conserved regions in the variable domain are called framework regions (FR). The CDRs of the light and heavy chains are primarily responsible for the interaction of the antibody with antigen. Numbering of amino acid positions used herein is according to the EU Index, as in See Kabat, E. A. *et al.* (1991) Sequences of Proteins of
10 Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242. In some embodiments, the variable region is a human variable region.

The term “expressed” or “expression” as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term “expressed” or “expression” as used herein
15 also refers to the translation from the RNA molecule to give a protein, a polypeptide or a portion thereof.

An mRNA that is “upregulated” is generally increased upon a given treatment or condition. An mRNA that is “downregulated” generally refers to a decrease in the level of expression of the mRNA in response to a given treatment or condition. In some situations, the
20 mRNA level can remain unchanged upon a given treatment or condition.

An mRNA from a patient sample can be “upregulated” when treated with a drug, as compared to a non-treated control. This upregulation can be, for example, an increase of about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 90%, 100%, 200%, 300%, 500%, 1,000%, 5,000% or more of the comparative control mRNA level.

25 Alternatively, an mRNA can be “downregulated”, or expressed at a lower level, in response to administration of certain compounds or other agents. A downregulated mRNA can be, for example, present at a level of about 99%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 1% or less of the comparative control mRNA level.

Similarly, the level of a polypeptide or protein biomarker from a patient sample can be
30 increased when treated with a drug, as compared to a non-treated control. This increase can be

about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 90%, 100%, 200%, 300%, 500%, 1,000%, 5,000% or more of the comparative control protein level.

Alternatively, the level of a protein biomarker can be decreased in response to administration of certain compounds or other agents. This decrease can be, for example, present
5 at a level of about 99%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 1% or less of the comparative control protein level.

The terms “determining”, “measuring”, “evaluating”, “assessing” and “assaying” as used herein generally refer to any form of measurement, and include determining if an element is present or not. These terms include both quantitative and/or qualitative determinations.

10 Assessing may be relative or absolute. “Assessing the presence of” can include determining the amount of something present, as well as determining whether it is present or absent.

The terms “nucleic acid” and “polynucleotide” are used interchangeably herein to describe a polymer of any length composed of nucleotides, *e.g.*, deoxyribonucleotides or ribonucleotides, or compounds produced synthetically, which can hybridize with naturally
15 occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, *e.g.*, can participate in Watson-Crick base pairing interactions. As used herein in the context of a polynucleotide sequence, the term “bases” (or “base”) is synonymous with “nucleotides” (or “nucleotide”), *i.e.*, the monomer subunit of a polynucleotide. The terms “nucleoside” and “nucleotide” are intended to include those moieties that contain not only the
20 known purine and pyrimidine bases, but also other heterocyclic bases that have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, alkylated riboses or other heterocycles. In addition, the terms “nucleoside” and “nucleotide” include those moieties that contain not only conventional ribose and deoxyribose sugars, but other sugars as well. Modified nucleosides or nucleotides also include modifications on the
25 sugar moiety, *e.g.*, wherein one or more of the hydroxyl groups are replaced with halogen atoms or aliphatic groups, or are functionalized as ethers, amines, or the like. “Analogues” refer to molecules having structural features that are recognized in the literature as being mimetics, derivatives, having analogous structures, or other like terms, and include, for example, polynucleotides incorporating non-natural nucleotides, nucleotide mimetics such as 2'-modified
30 nucleosides, peptide nucleic acids, oligomeric nucleoside phosphonates, and any polynucleotide that has added substituent groups, such as protecting groups or linking moieties.

The term “complementary” refers to specific binding between polynucleotides based on the sequences of the polynucleotides. As used herein, a first polynucleotide and a second polynucleotide are complementary if they bind to each other in a hybridization assay under stringent conditions, *e.g.* if they produce a given or detectable level of signal in a hybridization assay. Portions of polynucleotides are complementary to each other if they follow conventional base-pairing rules, *e.g.* A pairs with T (or U) and G pairs with C, although small regions (*e.g.* less than about 3 bases) of mismatch, insertion, or deleted sequence may be present.

“Sequence identity” or “identity” in the context of two nucleic acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window, and can take into consideration additions, deletions and substitutions.

The term “substantial identity” or “homologous” in their various grammatical forms in the context of polynucleotides generally means that a polynucleotide comprises a sequence that has a desired identity, for example, at least 60% identity, preferably at least 70% sequence identity, more preferably at least 80%, still more preferably at least 90% and even more preferably at least 95%, compared to a reference sequence. Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions.

The terms “isolated” and “purified” refer to isolation of a substance (such as mRNA, antibody or protein) such that the substance comprises a substantial portion of the sample in which it resides, *i.e.* greater than the substance is typically found in its natural or un-isolated state. Typically, a substantial portion of the sample comprises, *e.g.*, greater than 1%, greater than 2%, greater than 5%, greater than 10%, greater than 20%, greater than 50%, or more, usually up to about 90%-100% of the sample. For example, a sample of isolated mRNA can typically comprise at least about 1% total mRNA. Techniques for purifying polynucleotides are well known in the art and include, for example, gel electrophoresis, ion-exchange chromatography, affinity chromatography, flow sorting, and sedimentation according to density.

The term “sample” as used herein relates to a material or mixture of materials, typically, although not necessarily, in fluid form, containing one or more components of interest.

“Biological sample” as used herein refers to a sample obtained from a biological subject, including sample of biological tissue or fluid origin, obtained, reached, or collected *in vivo* or *in*

situ. A biological sample also includes samples from a region of a biological subject containing precancerous or cancer cells or tissues. Such samples can be, but are not limited to, organs, tissues, fractions and cells isolated from a mammal. Exemplary biological samples include but are not limited to cell lysate, a cell culture, a cell line, a tissue, oral tissue, gastrointestinal tissue, an organ, an organelle, a biological fluid, a blood sample, a urine sample, a skin sample, and the like. Preferred biological samples include but are not limited to whole blood, partially purified blood, PBMCs, tissue biopsies, and the like.

The term “capture agent,” as used herein, refers to an agent that binds an mRNA or protein through an interaction that is sufficient to permit the agent to bind and concentrate the mRNA or protein from a homogeneous mixture.

The term “probe” as used herein, refers to a capture agent that is directed to a specific target mRNA biomarker sequence. Accordingly, each probe of a probe set has a respective target mRNA biomarker. A probe/target mRNA duplex is a structure formed by hybridizing a probe to its target mRNA biomarker.

The term “nucleic acid” or “oligonucleotide probe” refers to a nucleic acid capable of binding to a target nucleic acid of complementary sequence, such as the mRNA biomarkers provided herein, through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*e.g.*, A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled with isotopes, for example, chromophores, lumiphores, chromogens, or indirectly labeled with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of a target mRNA biomarker of interest.

The term “stringent assay conditions” refers to conditions that are compatible to produce binding pairs of nucleic acids, *e.g.*, probes and target mRNAs, of sufficient complementarity to provide for the desired level of specificity in the assay while being generally incompatible to the formation of binding pairs between binding members of insufficient complementarity to provide

for the desired specificity. The term stringent assay conditions generally refers to the combination of hybridization and wash conditions.

A “label” or a “detectable moiety” in reference to a nucleic acid, refers to a composition that, when linked with a nucleic acid, renders the nucleic acid detectable, for example, by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Exemplary labels include, but are not limited to, radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, enzymes, biotin, digoxigenin, haptens, and the like. A “labeled nucleic acid or oligonucleotide probe” is generally one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic bonds, van der Waals forces, electrostatic attractions, hydrophobic interactions, or hydrogen bonds, to a label such that the presence of the nucleic acid or probe can be detected by detecting the presence of the label bound to the nucleic acid or probe.

The terms “Polymerase chain reaction,” or “PCR,” as used herein generally refers to a procedure wherein small amounts of a nucleic acid, RNA and/or DNA, are amplified as described, for example, in U.S. Pat. No. 4,683,195 to Mullis. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. *See generally* Mullis *et al.*, Cold Spring Harbor Symp. Quant. Biol., 51: 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989).

The term “cycle number” or “CT” when used herein in reference to PCR methods, refers to the PCR cycle number at which the fluorescence level passes a given set threshold level. The CT measurement can be used, for example, to approximate levels of mRNA in an original sample. The CT measurement is often used in terms of “dCT” or the “difference in the CT” score, when the CT of one nucleic acid is subtracted from the CT of another nucleic acid.

As used herein, and unless otherwise indicated, the term “optically pure” means a composition that comprises one optical isomer of a compound and is substantially free of other isomers of that compound. For example, an optically pure composition of a compound having

one chiral center will be substantially free of the opposite enantiomer of the compound. An optically pure composition of a compound having two chiral centers will be substantially free of other diastereomers of the compound. A typical optically pure compound comprises greater than about 80% by weight of one enantiomer of the compound and less than about 20% by weight of other enantiomers of the compound, more preferably greater than about 90% by weight of one enantiomer of the compound and less than about 10% by weight of the other enantiomers of the compound, even more preferably greater than about 95% by weight of one enantiomer of the compound and less than about 5% by weight of the other enantiomers of the compound, more preferably greater than about 97% by weight of one enantiomer of the compound and less than about 3% by weight of the other enantiomers of the compound, and most preferably greater than about 99% by weight of one enantiomer of the compound and less than about 1% by weight of the other enantiomers of the compound.

As used herein and unless otherwise indicated, the term “pharmaceutically acceptable salt” encompasses non-toxic acid and base addition salts of the compound to which the term refers.

Acceptable non-toxic acid addition salts include those derived from organic and inorganic acids or bases known in the art, which include, for example, hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid, methanesulphonic acid, acetic acid, tartaric acid, lactic acid, succinic acid, citric acid, malic acid, maleic acid, sorbic acid, aconitic acid, salicylic acid, phthalic acid, embolic acid, enanthic acid, and the like.

Compounds that are acidic in nature are capable of forming salts with various pharmaceutically acceptable bases. The bases that can be used to prepare pharmaceutically acceptable base addition salts of such acidic compounds are those that form non-toxic base addition salts, *i.e.*, salts containing pharmacologically acceptable cations such as, but not limited to, alkali metal or alkaline earth metal salts and the calcium, magnesium, sodium or potassium salts in particular. Suitable organic bases include, but are not limited to, N,N-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumaine (N-methylglucamine), lysine, and procaine.

As used herein and unless otherwise indicated, the term “solvate” means a compound provided herein or a salt thereof, that further includes a stoichiometric or non-stoichiometric amount of solvent bound by non-covalent intermolecular forces. Where the solvent is water, the solvate is a hydrate.

As used herein and unless otherwise indicated, the term “stereomerically pure” means a composition that comprises one stereoisomer of a compound and is substantially free of other stereoisomers of that compound. For example, a stereomerically pure composition of a compound having one chiral center will be substantially free of the opposite enantiomer of the compound. A stereomerically pure composition of a compound having two chiral centers will be substantially free of other diastereomers of the compound. A typical stereomerically pure compound comprises greater than about 80% by weight of one stereoisomer of the compound and less than about 20% by weight of other stereoisomers of the compound, more preferably greater than about 90% by weight of one stereoisomer of the compound and less than about 10% by weight of the other stereoisomers of the compound, even more preferably greater than about 95% by weight of one stereoisomer of the compound and less than about 5% by weight of the other stereoisomers of the compound, and most preferably greater than about 97% by weight of one stereoisomer of the compound and less than about 3% by weight of the other stereoisomers of the compound. As used herein and unless otherwise indicated, the term “stereomerically enriched” means a composition that comprises greater than about 60% by weight of one stereoisomer of a compound, preferably greater than about 70% by weight, more preferably greater than about 80% by weight of one stereoisomer of a compound. As used herein and unless otherwise indicated, the term “enantiomerically pure” means a stereomerically pure composition of a compound having one chiral center. Similarly, the term “stereomerically enriched” means a stereomerically enriched composition of a compound having one chiral center.

As used herein and unless otherwise indicated, the term “co-crystal” means a crystalline form that contains more than one compound in a crystal lattice. Co-crystals include crystalline molecular complexes of two or more non-volatile compounds bound together in a crystal lattice through non-ionic interactions. As used herein, co-crystals include pharmaceutical cocrystals wherein the crystalline molecular complexes contain a therapeutic compound and one or more additional non-volatile compound(s) (referred to herein as counter-molecule(s)). A counter-molecule in a pharmaceutical cocrystal is typically a non-toxic pharmaceutically acceptable molecule, such as, for example, food additives, preservatives, pharmaceutical excipients, or other APIs. In some embodiments, pharmaceutical cocrystals enhance certain physicochemical properties of drug products (*e.g.*, solubility, dissolution rate, bioavailability and/or stability) without compromising the chemical structural integrity of the active pharmaceutical ingredient

(API). See, e.g., Jones *et al.*, “Pharmaceutical Cocrystals: An Emerging Approach to Physical Property Enhancement,” *MRS Bulletin*, 2006, 31, 875–879; Trask, “An Overview of Pharmaceutical Cocrystals as Intellectual Property,” *Molecular Pharmaceutics*, 2007, 4(3), 301–309; Schultheiss & Newman, “Pharmaceutical Cocrystals and Their Physicochemical Properties,”
5 *Crystal Growth & Design*, 2009, 9(6), 2950–2967; Shan & Zaworotko, “The Role of Cocrystals in Pharmaceutical Science,” *Drug Discovery Today*, 2008, 13(9/10), 440–446; and Vishweshwar *et al.*, “Pharmaceutical Co-Crystals,” *J. Pharm. Sci.*, 2006, 95(3), 499–516.

A biological marker or “biomarker” is a substance whose detection indicates a particular biological state, such as, for example, the presence of cancer. In some embodiments, biomarkers
10 can either be determined individually, or several biomarkers can be measured simultaneously.

In some embodiments, a “biomarker” indicates a change in the level of mRNA expression that may correlate with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. In some embodiments, the biomarker is a nucleic acid, such as a mRNA or cDNA.

15 In additional embodiments, a “biomarker” indicates a change in the level of polypeptide or protein expression that may correlate with the risk, susceptibility to treatment, or progression of a disease. In some embodiments, the biomarker can be a polypeptide or protein, or a fragment thereof. The relative level of specific proteins can be determined by methods known in the art. For example, antibody based methods, such as an immunoblot, enzyme-linked immunosorbent
20 assay (ELISA), or other methods can be used.

It should be noted that if there is a discrepancy between a depicted structure and a name given that structure, the depicted structure is to be accorded more weight. In addition, if the stereochemistry of a structure or a portion of a structure is not indicated with, for example, bold or dashed lines, the structure or portion of the structure is to be interpreted as encompassing all
25 stereoisomers of it.

The practice of the embodiments provided herein will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and immunology, which are within the skill of those working in the art. Such techniques are explained fully in the literature. Examples of particularly suitable texts for consultation include the following: Sambrook *et al.*
30 (1989) *Molecular Cloning; A Laboratory Manual* (2d ed.); D.N Glover, ed. (1985) *DNA Cloning*, Volumes I and II; M.J. Gait, ed. (1984) *Oligonucleotide Synthesis*; B.D. Hames & S.J. Higgins,

eds. (1984) *Nucleic Acid Hybridization*; B.D. Hames & S.J. Higgins, eds. (1984) *Transcription and Translation*; R.I. Freshney, ed. (1986) *Animal Cell Culture; Immobilized Cells and Enzymes* (IRL Press, 1986); *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes (1987) *Protein Purification: Principles and Practice* (2d ed.; Springer Verlag, N.Y.); and D.M. Weir and C. C. Blackwell, eds. (1986) *Handbook of Experimental Immunology*, Volumes I-IV.

5.2 Clinical Trial Endpoints

“Overall survival” is defined as the time from randomization until death from any cause, and is measured in the intent-to-treat population. Overall survival should be evaluated in randomized controlled studies. Demonstration of a statistically significant improvement in overall survival can be considered to be clinically significant if the toxicity profile is acceptable, and has often supported new drug approval.

Several endpoints are based on cancer assessments. These endpoints include disease free survival (DFS), objective response rate (ORR), time to progression (TTP), progression-free survival (PFS), and time-to-treatment failure (TTF). The collection and analysis of data on these time-dependent endpoints are based on indirect assessments, calculations, and estimates (e.g., tumor measurements).

Generally, “disease free survival” (DFS) is defined as the time from randomization until recurrence of cancer or death from any cause. Although overall survival is a conventional endpoint for most adjuvant settings, DFS can be an important endpoint in situations where survival may be prolonged, making a survival endpoint impractical. DFS can be a surrogate for clinical benefit or it can provide direct evidence of clinical benefit. This determination is based on the magnitude of the effect, its risk-benefit relationship, and the disease setting. The definition of DFS can be complicated, particularly when deaths are noted without prior cancer progression documentation. These events can be scored either as disease recurrences or as censored events. Although all methods for statistical analysis of deaths have some limitations, considering all deaths (deaths from all causes) as recurrences can minimize bias. DFS can be overestimated using this definition, especially in patients who die after a long period without observation. Bias can be introduced if the frequency of long-term follow-up visits is dissimilar between the study arms or if dropouts are not random because of toxicity.

“Objective response rate” (ORR) is defined as the proportion of patients with cancer reduction of a predefined amount and for a minimum time period. Response duration usually is measured from the time of initial response until documented cancer progression. Generally, the FDA has defined ORR as the sum of partial responses plus complete responses. When defined in this manner, ORR is a direct measure of drug anticancer activity, which can be evaluated in a single-arm study. If available, standardized criteria should be used to ascertain response. A variety of response criteria have been considered appropriate (e.g., RECIST criteria) (Therasse et al., (2000) J. Natl. Cancer Inst, 92: 205-16). The significance of ORR is assessed by its magnitude and duration, and the percentage of complete responses (no detectable evidence of cancer).

“Time to progression” (TTP) and “progression-free survival” (PFS) have served as primary endpoints for drug approval. TTP is defined as the time from randomization until objective cancer progression; TTP does not include deaths. PFS is defined as the time from randomization until objective cancer progression or death. Compared with TTP, PFS is the preferred regulatory endpoint. PFS includes deaths and thus can be a better correlate to overall survival. PFS assumes patient deaths are randomly related to cancer progression. However, in situations where the majority of deaths are unrelated to cancer, TTP can be an acceptable endpoint.

As an endpoint to support drug approval, PFS can reflect cancer growth and be assessed before the determination of a survival benefit. Its determination is not confounded by subsequent therapy. For a given sample size, the magnitude of effect on PFS can be larger than the effect on overall survival. However, the formal validation of PFS as a surrogate for survival for the many different malignancies that exist can be difficult. Data are sometimes insufficient to allow a robust evaluation of the correlation between effects on survival and PFS. Cancer trials are often small, and proven survival benefits of existing drugs are generally modest. The role of PFS as an endpoint to support licensing approval varies in different cancer settings. Whether an improvement in PFS represents a direct clinical benefit or a surrogate for clinical benefit depends on the magnitude of the effect and the risk-benefit of the new treatment compared to available therapies.

“Time-to-treatment failure” (TTF) is defined as a composite endpoint measuring time from randomization to discontinuation of treatment for any reason, including disease progression, treatment toxicity, and death. TTF is not recommended as a regulatory endpoint for drug approval. TTF does not adequately distinguish efficacy from these additional variables. A

regulatory endpoint should clearly distinguish the efficacy of the drug from toxicity, patient or physician withdrawal, or patient intolerance.

5.3 Second Active Agents

The compounds provided herein may be combined with other pharmacologically active compounds (“second active agents”) in methods and compositions provided herein. It is believed that certain combinations work synergistically in the treatment of particular types of cancer, and certain diseases and conditions associated with or characterized by undesired angiogenesis and/or inflammation. The compounds provided herein provided herein can also work to alleviate adverse effects associated with certain second active agents, and some second active agents can be used to alleviate adverse effects associated with the compounds provided herein provided herein.

One or more second active ingredients or agents can be used in the methods and compositions provided herein with the compounds provided herein. Second active agents can be large molecules (*e.g.*, proteins) or small molecules (*e.g.*, synthetic inorganic, organometallic, or organic molecules).

Examples of large molecule active agents include, but are not limited to, hematopoietic growth factors, cytokines, and monoclonal and polyclonal antibodies. In certain embodiments, large molecule active agents are biological molecules, such as naturally occurring or artificially made proteins. Proteins that are particularly useful in this disclosure include proteins that stimulate the survival and/or proliferation of hematopoietic precursor cells and immunologically active poietic cells *in vitro* or *in vivo*. Others stimulate the division and differentiation of committed erythroid progenitors in cells *in vitro* or *in vivo*. Particular proteins include, but are not limited to: interleukins, such as IL-2 (including recombinant IL-II (“rIL2”) and canarypox IL-2), IL-10, IL-12, and IL-18; interferons, such as interferon alfa-2a, interferon alfa-2b, interferon alfa-n1, interferon alfa-n3, interferon beta-I a, and interferon gamma-I b; GM-CSF and GM-CSF; and EPO.

Particular proteins that can be used in the methods and compositions of the disclosure include, but are not limited to: filgrastim, which is sold in the United States under the trade name NEUPOGEN® (Amgen, Thousand Oaks, CA); sargramostim, which is sold in the United States under the trade name LEUKINE® (Immunex, Seattle, WA); and recombinant EPO, which is sold in the United States under the trade name EPOGEN® (Amgen, Thousand Oaks, CA).

Recombinant and mutated forms of GM-CSF can be prepared as described in U.S. Patent Nos. 5,391,485; 5,393,870; and 5,229,496; the disclosure of each of which is incorporated herein by reference in its entirety. Recombinant and mutated forms of G-CSF can be prepared as described in U.S. Patent Nos. 4,810,643; 4,999,291; 5,528,823; and 5,580,755; the disclosure of each of which is incorporated herein by reference in its entirety.

This disclosure encompasses the use of native, naturally occurring, and recombinant proteins. The disclosure further encompasses mutants and derivatives (*e.g.*, modified forms) of naturally occurring proteins that exhibit, *in vivo*, at least some of the pharmacological activity of the proteins upon which they are based. Examples of mutants include, but are not limited to, proteins that have one or more amino acid residues that differ from the corresponding residues in the naturally occurring forms of the proteins. Also encompassed by the term “mutants” are proteins that lack carbohydrate moieties normally present in their naturally occurring forms (*e.g.*, nonglycosylated forms). Examples of derivatives include, but are not limited to, pegylated derivatives and fusion proteins, such as proteins formed by fusing IgG1 or IgG3 to the protein or active portion of the protein of interest. *See, e.g.*, Penichet, M.L. and Morrison, S.L., *J. Immunol. Methods* 248:91-101 (2001).

Antibodies that can be used in combination with the compound of Formula I provided herein include monoclonal and polyclonal antibodies. Examples of antibodies include, but are not limited to, trastuzumab (HERCEPTIN[®]), rituximab (RITUXAN[®]), bevacizumab (AVASTIN[™]), pertuzumab (OMNITARG[™]), tositumomab (BEXXAR[®]), edrecolomab (PANOREX[®]), panitumumab and G250. The compound of Formula I provided herein can also be combined with or used in combination with anti-TNF- α antibodies.

Large molecule active agents may be administered in the form of anti-cancer vaccines. For example, vaccines that secrete, or cause the secretion of, cytokines such as IL-2, SCF, CXCL4 (platelet factor 4), G-CSF, and GM-CSF can be used in the methods, pharmaceutical compositions, and kits of the disclosure. *See, e.g.*, Emens, L.A., *et al.*, *Curr. Opinion Mol. Ther.* 3(1):77-84 (2001).

Second active agents that are small molecules can also be used to alleviate adverse effects associated with the administration of the compound of Formula I provided herein. However, like some large molecules, many are believed to be capable of providing a synergistic effect when administered with (*e.g.*, before, after or simultaneously) the compound of Formula I. Examples

of small molecule second active agents include, but are not limited to, anti-cancer agents, antibiotics, immunosuppressive agents, and steroids.

Examples of anti-cancer agents include, but are not limited to: abraxane; ace-111; acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; 5 ametantrone acetate; amrubicin; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropiramine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; celecoxib (COX-2 inhibitor); chlorambucil; cirolemycin; 10 cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin 15 hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; herceptin; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofofosine; iproplatin; irinotecan; irinotecan hydrochloride; 20 lanreotide acetate; lapatinib; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedopa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; 25 mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; romidepsin; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate 30 sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; stem cell treatments such as PDA-001; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan

sodium; taxotere; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; tricyriline phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulazole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinylicinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; and zorubicin hydrochloride.

Other anti-cancer drugs include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstauroporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; b-FGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; broprimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetorelix; chlorins; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentantraquinones; cycloplatam; cypemycin; cytarabine ocfosphate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrididemnin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziqune; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine; doxorubicin; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin;

epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole;
 etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride;
 flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorubicin hydrochloride; forfenimex;
 formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine;
 5 ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin;
 hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone;
 ilmofosine; ilomastat; imatinib (*e.g.*, GLEEVEC[®]), imiquimod; immunostimulant peptides;
 insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins;
 iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole;
 10 isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide;
 leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor;
 leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole;
 linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds;
 lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; loxoribine;
 15 lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A;
 marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors;
 menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone;
 miltefosine; mirimostim; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin
 fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; Erbitux, human
 20 chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol;
 mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone;
 N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine;
 napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; nilutamide;
 nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; oblimersen
 25 (GENASENSE[®]); O⁶-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone;
 ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin;
 oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine;
 palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine;
 pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozone; perflubron;
 30 perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil;
 pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator

inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins;

5 pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor

10 1; sense oligonucleotides; signal transduction inhibitors; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan

15 sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin; toremifene; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride;

20 tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vaporeotide; variolin B; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer.

Specific second active agents include, but are not limited to, oblimersen

25 (GENASENSE[®]), remicade, docetaxel, celecoxib, melphalan, dexamethasone (DECADRON[®]), steroids, gemcitabine, cisplatin, temozolomide, etoposide, cyclophosphamide, temodar, carboplatin, procarbazine, gliadel, tamoxifen, topotecan, methotrexate, ARISA[®], taxol, taxotere, fluorouracil, leucovorin, irinotecan, xeloda, CPT-11, interferon alpha, pegylated interferon alpha (e.g., PEG INTRON-A), capecitabine, cisplatin, thiotepa, fludarabine, carboplatin, liposomal

30 daunorubicin, cytarabine, doxetaxol, paclitaxel, vinblastine, IL-2, GM-CSF, dacarbazine, vinorelbine, zoledronic acid, palmitronate, biaxin, busulphan, prednisone, bisphosphonate,

arsenic trioxide, vincristine, doxorubicin (DOXIL[®]), paclitaxel, ganciclovir, adriamycin, estramustine sodium phosphate (EMCYT[®]), sulindac, and etoposide.

5.4 Methods of Treatment and Prevention

In one embodiment, provided herein is a method of treating and preventing cancer, which
5 comprises administering to a patient a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

In another embodiment, provided herein is method of managing cancer, which comprises administering to a patient a compound provided herein, or an enantiomer or a mixture of
10 enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

Also provided herein are methods of treating patients who have been previously treated for cancer but are non-responsive to standard therapies, as well as those who have not previously been treated. The invention also encompasses methods of treating patients regardless of patient's
15 age, although some diseases or disorders are more common in certain age groups. The invention further encompasses methods of treating patients who have undergone surgery in an attempt to treat the disease or condition at issue, as well as those who have not. Because patients with cancer have heterogeneous clinical manifestations and varying clinical outcomes, the treatment given to a patient may vary, depending on his/her prognosis. The skilled clinician will be able to
20 readily determine without undue experimentation specific secondary agents, types of surgery, and types of non-drug based standard therapy that can be effectively used to treat an individual patient with cancer.

In yet another embodiment, provided herein is a method of treating, managing, or preventing diseases and disorders other than cancer that are associated with or characterized by
25 undesired angiogenesis, which comprises administering to a patient a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

As used herein, the term "cancer" includes, but is not limited to, solid tumors and blood born tumors. The term "cancer" refers to disease of skin tissues, organs, blood, and vessels,
30 including, but not limited to, cancers of the bladder, bone, blood, brain, breast, cervix, chest, colon, endometrium, esophagus, eye, head, kidney, liver, lymph nodes, lung, mouth, neck,

ovaries, pancreas, prostate, rectum, stomach, testis, throat, and uterus. Specific cancers include, but are not limited to, advanced malignancy, amyloidosis, neuroblastoma, meningioma, hemangiopericytoma, multiple brain metastase, glioblastoma multiforms, glioblastoma, brain stem glioma, poor prognosis malignant brain tumor, malignant glioma, recurrent malignant
5 glioma, anaplastic astrocytoma, anaplastic oligodendroglioma, neuroendocrine tumor, rectal adenocarcinoma, Dukes C & D colorectal cancer, unresectable colorectal carcinoma, metastatic hepatocellular carcinoma, Kaposi's sarcoma, karotype acute myeloblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, cutaneous T-Cell lymphoma, cutaneous B-Cell lymphoma, diffuse large B-Cell lymphoma, low grade follicular lymphoma, malignant
10 melanoma, malignant mesothelioma, malignant pleural effusion mesothelioma syndrome, peritoneal carcinoma, papillary serous carcinoma, gynecologic sarcoma, soft tissue sarcoma, scleroderma, cutaneous vasculitis, Langerhans cell histiocytosis, leiomyosarcoma, fibrodysplasia ossificans progressive, hormone refractory prostate cancer, resected high-risk soft tissue sarcoma, unresectable hepatocellular carcinoma, Waldenstrom's macroglobulinemia, smoldering
15 myeloma, indolent myeloma, fallopian tube cancer, androgen independent prostate cancer, androgen dependent stage IV non-metastatic prostate cancer, hormone-insensitive prostate cancer, chemotherapy-insensitive prostate cancer, papillary thyroid carcinoma, follicular thyroid carcinoma, medullary thyroid carcinoma, and leiomyoma

In certain embodiments, the cancer is a blood borne tumor. In certain embodiments, the
20 blood borne tumor is metastatic. In certain embodiments, the blood borne tumor is drug resistant. In certain embodiments, the cancer is myeloma or lymphoma.

In certain embodiments, the cancer is a solid tumor. In certain embodiments, the solid tumor is metastatic. In certain embodiments, the solid tumor is drug-resistant. In certain
25 embodiments, the solid tumor is hepatocellular carcinoma, prostate cancer, ovarian cancer, or glioblastoma.

As used herein to refer to diseases and conditions other than cancer, the terms "diseases or disorders associated with or characterized by undesired angiogenesis," "diseases or disorders associated with undesired angiogenesis," and "diseases or disorders characterized by undesired angiogenesis" refer to diseases, disorders, and conditions that are caused, mediated, or attended
30 by undesired, unwanted, or uncontrolled angiogenesis, including, but not limited to, inflammatory diseases, autoimmune diseases, genetic diseases, allergic diseases, bacterial

diseases, ocular neovascular diseases, choroidal neovascular diseases, and retina neovascular diseases.

Examples of such diseases or disorders associated with undesired angiogenesis include, but are not limited to, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, 5 neovascular glaucoma, retrolental fibroplasia, proliferative vitreoretinopathy, trachoma, myopia, optic pits, epidemic keratoconjunctivitis, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phlyctenulosis, syphilis, lipid degeneration, bacterial ulcer, fungal ulcer, Herpes simplex infection, Herpes zoster infection, protozoan infection, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, 10 rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, sickle cell anemia, sarcoid, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis, chronic vitritis, Lyme's disease, Eales disease, Behcet's disease, retinitis, choroiditis, presumed ocular histoplasmosis, Bests disease, Stargarts disease, pars planitis, 15 chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, rubeosis, sarcodiosis, sclerosis, soriatis, psoriasis, primary sclerosing cholangitis, proctitis, primary biliary srosis, idiopathic pulmonary fibrosis, and alcoholic hepatitis.

In certain embodiments, a therapeutically or prophylactically effective amount of the compound is from about 0.005 to about 1,000 mg per day, from about 0.01 to about 500 mg per 20 day, from about 0.01 to about 250 mg per day, from about 0.01 to about 100 mg per day, from about 0.1 to about 100 mg per day, from about 0.5 to about 100 mg per day, from about 1 to about 100 mg per day, from about 0.01 to about 50 mg per day, from about 0.1 to about 50 mg per day, from about 0.5 to about 50 mg per day, from about 1 to about 50 mg per day, from about 0.02 to about 25 mg per day, or from about 0.05 to about 10 mg per day.

25 In certain embodiments, the therapeutically or prophylactically effective amount is about 1, about 2, about 5, about 10, about 15, about 20, about 25, about 30, about 40, about 45, about 50, about 60, about 70, about 80, about 90, about 100, or about 150 mg per day.

In one embodiment, the recommended daily dose range of the compound for the conditions described herein lie within the range of from about 0.5 mg to about 50 mg per day, preferably 30 given as a single once-a-day dose, or in divided doses throughout a day. In some embodiments, the dosage ranges from about 1 mg to about 50 mg per day. In other embodiments, the dosage

ranges from about 0.5 to about 5 mg per day. Specific doses per day include 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 mg per day. In other embodiments, specific doses per day include 0.5, 1, 1.5, 2, 2.5, 3, 3.5 or 4 mg per day.

5 In a specific embodiment, the recommended starting dosage may be 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 10, 15, 20, 25 or 50 mg per day. In another embodiment, the recommended starting dosage may be 0.5, 1, 1.5, 2, 2.5, 3, 3.5 or 4 mg per day. The dose may be escalated to 15, 20, 25, 30, 35, 40, 45 and 50 mg/day. In a specific embodiment, the compound can be administered in an amount of about 25 mg/day to patients with cancer. In a particular embodiment, the compound can be
10 administered in an amount of about 10 mg/day to patients with cancer.

In certain embodiments, the therapeutically or prophylactically effective amount is from about 0.001 to about 100 mg/kg/day, from about 0.01 to about 50 mg/kg/day, from about 0.01 to about 25 mg/kg/day, from about 0.01 to about 10 mg/kg/day, from about 0.01 to about 9 mg/kg/day, 0.01 to about 8 mg/kg/day, from about 0.01 to about 7 mg/kg/day, from about 0.01 to
15 about 6 mg/kg/day, from about 0.01 to about 5 mg/kg/day, from about 0.01 to about 4 mg/kg/day, from about 0.01 to about 3 mg/kg/day, from about 0.01 to about 2 mg/kg/day, or from about 0.01 to about 1 mg/kg/day.

The administered dose can also be expressed in units other than mg/kg/day. For example, doses for parenteral administration can be expressed as mg/m²/day. One of ordinary skill in the
20 art would readily know how to convert doses from mg/kg/day to mg/m²/day to given either the height or weight of a subject or both (*see*, www.fda.gov/cder/cancer/animalframe.htm). For example, a dose of 1 mg/kg/day for a 65 kg human is approximately equal to 38 mg/m²/day.

In certain embodiments, the amount of the compound administered is sufficient to provide a plasma concentration of the compound at steady state, ranging from about 0.001 to
25 about 500 μM, about 0.002 to about 200 μM, about 0.005 to about 100 μM, about 0.01 to about 50 μM, from about 1 to about 50 μM, about 0.02 to about 25 μM, from about 0.05 to about 20 μM, from about 0.1 to about 20 μM, from about 0.5 to about 20 μM, or from about 1 to about 20 μM.

In other embodiments, the amount of the compound administered is sufficient to provide
30 a plasma concentration of the compound at steady state, ranging from about 5 to about 100 nM,

about 5 to about 50 nM, about 10 to about 100 nM, about 10 to about 50 nM or from about 50 to about 100 nM.

As used herein, the term “plasma concentration at steady state” is the concentration reached after a period of administration of a compound provided herein, *e.g.*, the compound of Formula I, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof. Once steady state is reached, there are minor peaks and troughs on the time dependent curve of the plasma concentration of the compound.

In certain embodiments, the amount of the compound administered is sufficient to provide a maximum plasma concentration (peak concentration) of the compound, ranging from about 0.001 to about 500 μM , about 0.002 to about 200 μM , about 0.005 to about 100 μM , about 0.01 to about 50 μM , from about 1 to about 50 μM , about 0.02 to about 25 μM , from about 0.05 to about 20 μM , from about 0.1 to about 20 μM , from about 0.5 to about 20 μM , or from about 1 to about 20 μM .

In certain embodiments, the amount of the compound administered is sufficient to provide a minimum plasma concentration (trough concentration) of the compound, ranging from about 0.001 to about 500 μM , about 0.002 to about 200 μM , about 0.005 to about 100 μM , about 0.01 to about 50 μM , from about 1 to about 50 μM , about 0.01 to about 25 μM , from about 0.01 to about 20 μM , from about 0.02 to about 20 μM , from about 0.02 to about 20 μM , or from about 0.01 to about 20 μM .

In certain embodiments, the amount of the compound administered is sufficient to provide an area under the curve (AUC) of the compound, ranging from about 100 to about 100,000 $\text{ng}\cdot\text{hr}/\text{mL}$, from about 1,000 to about 50,000 $\text{ng}\cdot\text{hr}/\text{mL}$, from about 5,000 to about 25,000 $\text{ng}\cdot\text{hr}/\text{mL}$, or from about 5,000 to about 10,000 $\text{ng}\cdot\text{hr}/\text{mL}$.

In certain embodiments, the patient to be treated with one of the methods provided herein has not been treated with anticancer therapy prior to the administration of the drug. In certain embodiments, the patient to be treated with one of the methods provided herein has been treated with anticancer therapy prior to the administration of the drug. In certain embodiments, the patient to be treated with one of the methods provided herein has developed drug resistance to the anticancer therapy.

The methods provided herein encompass treating a patient regardless of patient's age, although some diseases or disorders are more common in certain age groups. Further provided herein is a method for treating a patient who has undergone surgery in an attempt to treat the disease or condition at issue, as well in one who has not. Because the subjects with cancer have heterogeneous clinical manifestations and varying clinical outcomes, the treatment given to a particular subject may vary, depending on his/her prognosis. The skilled clinician will be able to readily determine without undue experimentation, specific secondary agents, types of surgery, and types of non-drug based standard therapy that can be effectively used to treat an individual subject with cancer.

Depending on the disease to be treated and the subject's condition, the compound provided herein, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, may be administered by oral, parenteral (e.g., intramuscular, intraperitoneal, intravenous, CIV, intracisternal injection or infusion, subcutaneous injection, or implant), inhalation, nasal, vaginal, rectal, sublingual, or topical (e.g., transdermal or local) routes of administration. The compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, may be formulated, alone or together, in suitable dosage unit with pharmaceutically acceptable excipients, carriers, adjuvants and vehicles, appropriate for each route of administration.

In one embodiment, the compound provided herein, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered orally. In another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered parenterally. In yet another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered intravenously.

The compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, can be delivered as a single dose such as, e.g., a single bolus injection, or oral tablets or pills; or over time, such as, e.g., continuous infusion over time or divided bolus doses over time. The

compound can be administered repeatedly if necessary, for example, until the patient experiences stable disease or regression, or until the patient experiences disease progression or unacceptable toxicity. For example, stable disease for solid tumors generally means that the perpendicular diameter of measurable lesions has not increased by 25% or more from the last measurement.

5 Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines, *Journal of the National Cancer Institute* 92(3): 205-216 (2000). Stable disease or lack thereof is determined by methods known in the art such as evaluation of patient symptoms, physical examination, visualization of the tumor that has been imaged using X-ray, CAT, PET, or MRI scan and other commonly accepted evaluation modalities.

10 The compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, can be administered once daily (QD), or divided into multiple daily doses such as twice daily (BID), three times daily (TID), and four times daily (QID). In addition, the administration can be continuous (*i.e.*, daily for consecutive days or every day), intermittent, *e.g.*, in cycles (*i.e.*,
15 including days, weeks, or months of rest without drug). As used herein, the term “daily” is intended to mean that a therapeutic compound is administered once or more than once each day, for example, for a period of time. The term “continuous” is intended to mean that a therapeutic compound is administered daily for an uninterrupted period of at least 10 days to 52 weeks. The term “intermittent” or “intermittently” as used herein is intended to mean stopping and starting at
20 either regular or irregular intervals. For example, intermittent administration of a compound is administration for one to six days per week, administration in cycles (*e.g.*, daily administration for two to eight consecutive weeks, then a rest period with no administration for up to one week), or administration on alternate days. The term “cycling” as used herein is intended to mean that a therapeutic compound is administered daily or continuously but with a rest period.

25 In some embodiments, the frequency of administration is in the range of about a daily dose to about a monthly dose. In certain embodiments, administration is once a day, twice a day, three times a day, four times a day, once every other day, twice a week, once every week, once every two weeks, once every three weeks, or once every four weeks. In one embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically
30 acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered once a day. In another embodiment, the compound, or an enantiomer or a mixture of enantiomers

thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered twice a day. In yet another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered three times a day. In still
5 another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered four times a day.

In certain embodiments, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or
10 polymorph thereof, is administered once per day from one day to six months, from one week to three months, from one week to four weeks, from one week to three weeks, or from one week to two weeks. In certain embodiments, the compound, or a pharmaceutically acceptable salt or solvate thereof, is administered once per day for one week, two weeks, three weeks, or four weeks. In one embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof;
15 or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered once per day for one week. In another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered once per day for two weeks. In yet another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof;
20 or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered once per day for three weeks. In still another embodiment, the compound, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered once per day for four weeks.

25 **5.5 Combination Therapy With A Second Active Agent**

A compound provided herein, or an enantiomer or a mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, can also be combined or used in combination with other therapeutic agents useful in the treatment and/or prevention of a disease described herein.

30 In one embodiment, provided herein is a method of treating, preventing, or managing cancer, comprising administering to a patient compound provided herein, or an enantiomer or a

mixture of enantiomers thereof; or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof; in combination with one or more second active agents, and optionally in combination with radiation therapy, blood transfusions, or surgery. Examples of second active agents are disclosed herein (*see, e.g.*, section 5.3).

5 As used herein, the term “in combination” includes the use of more than one therapy (e.g., one or more prophylactic and/or therapeutic agents). However, the use of the term “in combination” does not restrict the order in which therapies (e.g., prophylactic and/or therapeutic agents) are administered to a patient with a disease or disorder. A first therapy (e.g., a prophylactic or therapeutic agent such as a compound provided herein, a compound provided
10 herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof) can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes,
15 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy (e.g., a prophylactic or therapeutic agent) to the subject. Triple therapy is also contemplated herein.

Administration of the compound and one or more second active agents to a patient can
20 occur simultaneously or sequentially by the same or different routes of administration. The suitability of a particular route of administration employed for a particular active agent will depend on the active agent itself (*e.g.*, whether it can be administered orally without decomposing prior to entering the blood stream) and the cancer being treated.

The route of administration of the compound is independent of the route of
25 administration of a second therapy. In one embodiment, the compound is administered orally. In another embodiment, the compound is administered intravenously. Thus, in accordance with these embodiments, the compound is administered orally or intravenously, and the second therapy can be administered orally, parenterally, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally,
30 via inhalation, vaginally, intraocularly, via local delivery by catheter or stent, subcutaneously, intraadiposally, intraarticularly, intrathecally, or in a slow release dosage form. In one

embodiment, the compound and a second therapy are administered by the same mode of administration, orally or by IV. In another embodiment, the compound is administered by one mode of administration, e.g., by IV, whereas the second agent (an anticancer agent) is administered by another mode of administration, e.g., orally.

5 In one embodiment, the second active agent is administered intravenously or subcutaneously and once or twice daily in an amount of from about 1 to about 1000 mg, from about 5 to about 500 mg, from about 10 to about 350 mg, or from about 50 to about 200 mg. The specific amount of the second active agent will depend on the specific agent used, the type of disease being treated or managed, the severity and stage of disease, and the amount of the
10 drug provided herein and any optional additional active agents concurrently administered to the patient. In certain embodiments, the second active agent is oblimersen (GENASENSE[®]), GM-CSF, G-CSF, SCF, EPO, taxotere, irinotecan, dacarbazine, transretinoic acid, topotecan, pentoxifylline, ciprofloxacin, dexamethasone, vincristine, doxorubicin, COX-2 inhibitor, IL2, IL8, IL18, IFN, Ara-C, vinorelbine, or a combination thereof.

15 In certain embodiments, GM-CSF, G-CSF, SCF or EPO is administered subcutaneously during about five days in a four or six week cycle in an amount ranging from about 1 to about 750 mg/m²/day, from about 25 to about 500 mg/m²/day, from about 50 to about 250 mg/m²/day, or from about 50 to about 200 mg/m²/day. In certain embodiments, GM-CSF may be administered in an amount of from about 60 to about 500 mcg/m² intravenously over 2 hours or
20 from about 5 to about 12 mcg/m²/day subcutaneously. In certain embodiments, G-CSF may be administered subcutaneously in an amount of about 1 mcg/kg/day initially and can be adjusted depending on rise of total granulocyte counts. The maintenance dose of G-CSF may be administered in an amount of about 300 (in smaller patients) or 480 mcg subcutaneously. In certain embodiments, EPO may be administered subcutaneously in an amount of 10,000 Unit 3
25 times per week.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with melphalan and dexamethasone to patients with amyloidosis. In certain embodiments, a compound provided herein, e.g., the compound of
30 Formula I, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically

acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, and steroids can be administered to patients with amyloidosis.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with gemcitabine and cisplatinum to patients with locally
5 advanced or metastatic transitional cell bladder cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with a second active ingredient as follows:
10 temozolomide to pediatric patients with relapsed or progressive brain tumors or recurrent neuroblastoma; celecoxib, etoposide and cyclophosphamide for relapsed or progressive CNS cancer; temodar to patients with recurrent or progressive meningioma, malignant meningioma, hemangiopericytoma, multiple brain metastases, relapsed brain tumors, or newly diagnosed glioblastoma multiforms; irinotecan to patients with recurrent glioblastoma; carboplatin to
15 pediatric patients with brain stem glioma; procarbazine to pediatric patients with progressive malignant gliomas; cyclophosphamide to patients with poor prognosis malignant brain tumors, newly diagnosed or recurrent glioblastoma multiforms; Gliadel[®] for high grade recurrent malignant gliomas; temozolomide and tamoxifen for anaplastic astrocytoma; or topotecan for gliomas, glioblastoma, anaplastic astrocytoma or anaplastic oligodendroglioma.

20 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with methotrexate, cyclophosphamide, taxane, abraxane, lapatinib, herceptin, aromatase inhibitors, selective estrogen modulators, estrogen receptor antagonists, and/or PLX3397 (Plexxikon) to patients with metastatic breast cancer.

25 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with temozolomide to patients with neuroendocrine tumors.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of
30 enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate,

or polymorph thereof, is administered with gemcitabine to patients with recurrent or metastatic head or neck cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with gemcitabine to patients with pancreatic cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with colon cancer in combination with ARISA[®], avastatin, taxol, and/or taxotere.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with capecitabine and/or PLX4032 (Plexxikon) to patients with refractory colorectal cancer or patients who fail first line therapy or have poor performance in colon or rectal adenocarcinoma.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with fluorouracil, leucovorin, and irinotecan to patients with Dukes C & D colorectal cancer or to patients who have been previously treated for metastatic colorectal cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with refractory colorectal cancer in combination with capecitabine, xeloda, and/or CPT-11.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with capecitabine and irinotecan to patients with refractory colorectal cancer or to patients with unresectable or metastatic colorectal carcinoma.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered alone or in combination with interferon alpha or

capecitabine to patients with unresectable or metastatic hepatocellular carcinoma; or with cisplatin and thiotepa to patients with primary or metastatic liver cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with pegylated interferon alpha to patients with Kaposi's sarcoma.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with fludarabine, carboplatin, and/or topotecan to patients with refractory or relapsed or high-risk acuted myelogenous leukemia.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with liposomal daunorubicin, topotecan and/or cytarabine to patients with unfavorable karotype acute myeloblastic leukemia.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with gemcitabine, abraxane, erlotinib, gefitinib, and/or irinotecan to patients with non-small cell lung cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with carboplatin and irinotecan to patients with non-small cell lung cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered with doxorubicin to patients with non-small cell lung cancer who have been previously treated with carbo/VP 16 and radiotherapy.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with carboplatin and/or taxotere, or in combination with carboplatin, paclitaxel and/or thoracic radiotherapy to patients with non-small cell lung cancer.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with taxotere to patients with stage IIIB or IV non-small cell lung cancer.

5 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with oblimersen (Genasense[®]) to patients with small cell lung cancer.

10 In certain embodiments, a compound provided herein, *e.g.*, the compound of Formula I, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with ABT-737 (Abbott Laboratories) and/or obatoclox (GX15-070) to patients with lymphoma and other blood cancers.

15 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered alone or in combination with a second active ingredient such as vinblastine or fludarabine to patients with various types of lymphoma, including, but not limited to, Hodgkin's lymphoma, non-Hodgkin's lymphoma, cutaneous T-Cell lymphoma, cutaneous B-Cell lymphoma, diffuse large B-Cell lymphoma or relapsed or refractory low grade
20 follicular lymphoma.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with taxotere, IL-2, IFN, GM-CSF, PLX4032 (Plexxikon) and/or dacarbazine to patients with various types or stages of melanoma.

25 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered alone or in combination with vinorelbine to patients with malignant mesothelioma, or stage IIIB non-small cell lung cancer with pleural implants or malignant pleural effusion mesothelioma syndrome.

30 In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate,

or polymorph thereof, is administered to patients with various types or stages of multiple myeloma in combination with dexamethasone, zoledronic acid, palmitronate, GM-CSF, biacin, vinblastine, melphalan, busulphan, cyclophosphamide, IFN, palmidronate, prednisone, bisphosphonate, celecoxib, arsenic trioxide, PEG INTRON-A, vincristine, or a combination thereof.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with relapsed or refractory multiple myeloma in combination with doxorubicin (Doxil[®]), vincristine and/or dexamethasone (Decadron[®]).

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with various types or stages of ovarian cancer such as peritoneal carcinoma, papillary serous carcinoma, refractory ovarian cancer or recurrent ovarian cancer, in combination with taxol, carboplatin, doxorubicin, gemcitabine, cisplatin, xeloda, paclitaxel, dexamethasone, or a combination thereof.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with various types or stages of prostate cancer, in combination with xeloda, 5 FU/LV, gemcitabine, irinotecan plus gemcitabine, cyclophosphamide, vincristine, dexamethasone, GM-CSF, celecoxib, taxotere, ganciclovir, paclitaxel, adriamycin, docetaxel, estramustine, Emcyt, denderon or a combination thereof.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with various types or stages of renal cell cancer, in combination with capecitabine, IFN, tamoxifen, IL-2, GM-CSF, Celebrex[®], or a combination thereof.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with various types or stages of gynecologic, uterus or soft tissue sarcoma cancer in combination with IFN, a COX-2 inhibitor such as Celebrex[®], and/or sulindac.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with various types or stages of solid tumors in combination with celebrex, etoposide, cyclophosphamide, docetaxel, apicitabine, IFN, tamoxifen, IL-2, GM-CSF, or a combination thereof.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with scleroderma or cutaneous vasculitis in combination with celebrex, etoposide, cyclophosphamide, docetaxel, apicitabine, IFN, tamoxifen, IL-2, GM-CSF, or a combination thereof.

Also encompassed herein is a method of increasing the dosage of an anti-cancer drug or agent that can be safely and effectively administered to a patient, which comprises administering to the patient (*e.g.*, a human) or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

Patients that can benefit by this method are those likely to suffer from an adverse effect associated with anti-cancer drugs for treating a specific cancer of the skin, subcutaneous tissue, lymph nodes, brain, lung, liver, bone, intestine, colon, heart, pancreas, adrenal, kidney, prostate, breast, colorectal, or combinations thereof. The administration of a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, alleviates or reduces adverse effects which are of such severity that it would otherwise limit the amount of anti-cancer drug.

In one embodiment, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered orally and daily in an amount ranging from about 0.1 to about 150 mg, from about 1 to about 50 mg, or from about 2 to about 25 mg, prior to, during, or after the occurrence of the adverse effect associated with the administration of an anti-cancer drug to a patient. In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered in combination with specific agents such as heparin, aspirin, coumadin, or G-CSF to avoid adverse effects that are associated with anti-cancer drugs such as but not limited to neutropenia or thrombocytopenia.

In one embodiment, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, is administered to patients with diseases and disorders associated with or characterized by, undesired angiogenesis in combination with additional active ingredients,
5 including, but not limited to, anti-cancer drugs, anti-inflammatories, antihistamines, antibiotics, and steroids.

In another embodiment, encompassed herein is a method of treating, preventing and/or managing cancer, which comprises administering the compound, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal,
10 clathrate, or polymorph thereof, in conjunction with (*e.g.* before, during, or after) conventional therapy including, but not limited to, surgery, immunotherapy, biological therapy, radiation therapy, or other non-drug based therapy presently used to treat, prevent or manage cancer. The combined use of the compound provided herein and conventional therapy may provide a unique treatment regimen that is unexpectedly effective in certain patients. Without being limited by
15 theory, it is believed that the compound of Formula I may provide additive or synergistic effects when given concurrently with conventional therapy.

As discussed elsewhere herein, encompassed herein is a method of reducing, treating and/or preventing adverse or undesired effects associated with conventional therapy including, but not limited to, surgery, chemotherapy, radiation therapy, hormonal therapy, biological
20 therapy and immunotherapy. A compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof, and other active ingredient can be administered to a patient prior to, during, or after the occurrence of the adverse effect associated with conventional therapy.

In one embodiment, the compound can be administered in an amount ranging from about
25 0.1 to about 150 mg, from about 1 to about 25 mg, from about 2 to about 10 mg, or about 0.5 to about 4 mg orally and daily alone, or in combination with a second active agent disclosed herein (*see, e.g.*, section 4.3), prior to, during, or after the use of conventional therapy.

In certain embodiments, a compound provided herein, or an enantiomer or a mixture of enantiomers thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate,
30 or polymorph thereof, and doxorubicin are administered to patients with non-small cell lung cancer who were previously treated with carbo/VP 16 and radiotherapy.

5.6 Pharmaceutical Compositions

Pharmaceutical compositions can be used in the preparation of individual, single unit dosage forms. Pharmaceutical compositions and dosage forms provided herein comprise a compound, or a pharmaceutically acceptable salt, solvate, hydrate, stereoisomer, clathrate, or
5 prodrug thereof. Pharmaceutical compositions and dosage forms provided herein may further comprise one or more excipients.

Pharmaceutical compositions and dosage forms provided herein may also comprise one or more additional active ingredients. Examples of optional second, or additional, active ingredients are disclosed herein.

10 Single unit dosage forms are suitable for oral, mucosal (*e.g.*, nasal, sublingual, vaginal, buccal, or rectal), parenteral (*e.g.*, subcutaneous, intravenous, bolus injection, intramuscular, or intraarterial), topical (*e.g.*, eye drops or other ophthalmic preparations), transdermal or transcutaneous administration to a patient. Examples of dosage forms include, but are not limited to: tablets; caplets; capsules, such as soft elastic gelatin capsules; cachets; troches;
15 lozenges; dispersions; suppositories; powders; aerosols (*e.g.*, nasal sprays or inhalers); gels; liquid dosage forms suitable for oral or mucosal administration to a patient, including suspensions (*e.g.*, aqueous or non-aqueous liquid suspensions, oil-in-water emulsions, or a water-in-oil liquid emulsions), solutions, and elixirs; liquid dosage forms suitable for parenteral administration to a patient; eye drops or other ophthalmic preparations suitable for topical
20 administration; and sterile solids (*e.g.*, crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient.

The composition, shape, and type of dosage forms provided herein will typically vary depending on their use. For example, a dosage form used in the acute treatment of a disease may contain larger amounts of one or more of the active ingredients it comprises than a dosage form
25 used in the chronic treatment of the same disease. Similarly, a parenteral dosage form may contain smaller amounts of one or more of the active ingredients it comprises than an oral dosage form used to treat the same disease. These and other ways in which specific dosage forms provided herein will vary from one another will be readily apparent to those skilled in the art. *See, e.g., Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing, Easton PA (1990).

30 Typical pharmaceutical compositions and dosage forms comprise one or more excipients. Suitable excipients are well known to those skilled in the art of pharmacy, and non-limiting

examples of suitable excipients are provided herein. Whether a particular excipient is suitable for incorporation into a pharmaceutical composition or dosage form depends on a variety of factors well known in the art including, but not limited to, the way in which the dosage form will be administered to a patient. For example, oral dosage forms such as tablets may contain
5 excipients not suited for use in parenteral dosage forms. The suitability of a particular excipient may also depend on the specific active ingredients in the dosage form. For example, the decomposition of some active ingredients may be accelerated by some excipients such as lactose, or when exposed to water. Active ingredients that comprise primary or secondary amines are particularly susceptible to such accelerated decomposition. Consequently, provided herein are
10 pharmaceutical compositions and dosage forms that contain little, if any, lactose other mono- or di-saccharides. As used herein, the term "lactose-free" means that the amount of lactose present, if any, is insufficient to substantially increase the degradation rate of an active ingredient.

Lactose-free compositions provided herein can comprise excipients that are well known in the art and are listed, for example, in the *U.S. Pharmacopeia* (USP) 25-NF20 (2002). In
15 general, lactose-free compositions comprise active ingredients, a binder/filler, and a lubricant in pharmaceutically compatible and pharmaceutically acceptable amounts. In one embodiment, lactose-free dosage forms comprise active ingredients, microcrystalline cellulose, pre-gelatinized starch, and magnesium stearate.

Also provided herein are anhydrous pharmaceutical compositions and dosage forms
20 comprising active ingredients, since water can facilitate the degradation of some compounds. For example, the addition of water (*e.g.*, 5%) is widely accepted in the pharmaceutical arts as a means of simulating long-term storage in order to determine characteristics such as shelf-life or the stability of formulations over time. *See, e.g.*, Jens T. Carstensen, *Drug Stability: Principles & Practice*, 2d. Ed., Marcel Dekker, NY, NY, 1995, pp. 379-80. In effect, water and heat
25 accelerate the decomposition of some compounds. Thus, the effect of water on a formulation can be of great significance since moisture and/or humidity are commonly encountered during manufacture, handling, packaging, storage, shipment, and use of formulations.

Anhydrous pharmaceutical compositions and dosage forms may be prepared using anhydrous or low moisture containing ingredients and low moisture or low humidity conditions.
30 Pharmaceutical compositions and dosage forms that comprise lactose and at least one active ingredient that comprises a primary or secondary amine are preferably anhydrous if substantial

contact with moisture and/or humidity during manufacturing, packaging, and/or storage is expected.

An anhydrous pharmaceutical composition should be prepared and stored such that its anhydrous nature is maintained. Accordingly, anhydrous compositions are preferably packaged
5 using materials known to prevent exposure to water such that they can be included in suitable formulary kits. Examples of suitable packaging include, but are not limited to, hermetically sealed foils, plastics, unit dose containers (*e.g.*, vials), blister packs, and strip packs.

Also provided herein are pharmaceutical compositions and dosage forms that comprise one or more compounds that reduce the rate by which an active ingredient will decompose. Such
10 compounds, which are referred to herein as “stabilizers,” include, but are not limited to, antioxidants such as ascorbic acid, pH buffers, or salt buffers.

5.7 Oral Dosage Forms

Pharmaceutical compositions that are suitable for oral administration can be presented as discrete dosage forms, such as, but are not limited to, tablets (*e.g.*, chewable tablets), caplets,
15 capsules, and liquids (*e.g.*, flavored syrups). Such dosage forms contain predetermined amounts of active ingredients, and may be prepared by methods of pharmacy well known to those skilled in the art. *See generally, Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing, Easton PA (1990).

Typical oral dosage forms are prepared by combining the active ingredients in an
20 intimate admixture with at least one excipient according to conventional pharmaceutical compounding techniques. Excipients can take a wide variety of forms depending on the form of preparation desired for administration. For example, excipients suitable for use in oral liquid or aerosol dosage forms include, but are not limited to, water, glycols, oils, alcohols, flavoring agents, preservatives, and coloring agents. Examples of excipients suitable for use in solid oral
25 dosage forms (*e.g.*, powders, tablets, capsules, and caplets) include, but are not limited to, starches, sugars, micro-crystalline cellulose, diluents, granulating agents, lubricants, binders, and disintegrating agents.

Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit forms, in which case solid excipients are employed. If desired,
30 tablets can be coated by standard aqueous or nonaqueous techniques. Such dosage forms can be prepared by any of the methods of pharmacy. In general, pharmaceutical compositions and

dosage forms are prepared by uniformly and intimately admixing the active ingredients with liquid carriers, finely divided solid carriers, or both, and then shaping the product into the desired presentation if necessary.

For example, a tablet can be prepared by compression or molding. Compressed tablets
5 can be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as powder or granules, optionally mixed with an excipient. Molded tablets can be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

Examples of excipients that can be used in oral dosage forms provided herein include, but
10 are not limited to, binders, fillers, disintegrants, and lubricants. Binders suitable for use in pharmaceutical compositions and dosage forms include, but are not limited to, corn starch, potato starch, or other starches, gelatin, natural and synthetic gums such as acacia, sodium alginate, alginic acid, other alginates, powdered tragacanth, guar gum, cellulose and its derivatives (*e.g.*, ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl
15 cellulose), polyvinyl pyrrolidone, methyl cellulose, pre-gelatinized starch, hydroxypropyl methyl cellulose, (*e.g.*, Nos. 2208, 2906, 2910), microcrystalline cellulose, and mixtures thereof.

Suitable forms of microcrystalline cellulose include, but are not limited to, the materials sold as AVICEL-PH-101, AVICEL-PH-103 AVICEL RC-581, AVICEL-PH-105 (available from FMC Corporation, American Viscose Division, Avicel Sales, Marcus Hook, PA), and
20 mixtures thereof. An specific binder is a mixture of microcrystalline cellulose and sodium carboxymethyl cellulose sold as AVICEL RC-581. Suitable anhydrous or low moisture excipients or additives include AVICEL-PH-103™ and Starch 1500 LM.

Examples of fillers suitable for use in the pharmaceutical compositions and dosage forms disclosed herein include, but are not limited to, talc, calcium carbonate (*e.g.*, granules or powder),
25 microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol, starch, pre-gelatinized starch, and mixtures thereof. The binder or filler in pharmaceutical compositions of the invention is typically present in from about 50 to about 99 weight percent of the pharmaceutical composition or dosage form.

Disintegrants are used in compositions to provide tablets that disintegrate when exposed
30 to an aqueous environment. Tablets that contain too much disintegrant may disintegrate in storage, while those that contain too little may not disintegrate at a desired rate or under the

desired conditions. Thus, a sufficient amount of disintegrant that is neither too much nor too little to detrimentally alter the release of the active ingredients should be used to form solid oral dosage forms. The amount of disintegrant used varies based upon the type of formulation, and is readily discernible to those of ordinary skill in the art. Typical pharmaceutical compositions
5 comprise from about 0.5 to about 15 weight percent of disintegrant, preferably from about 1 to about 5 weight percent of disintegrant.

Disintegrants that can be used in pharmaceutical compositions and dosage forms include, but are not limited to, agar-agar, alginic acid, calcium carbonate, microcrystalline cellulose, croscarmellose sodium, crospovidone, polacrillin potassium, sodium starch glycolate, potato or
10 tapioca starch, other starches, pre-gelatinized starch, other starches, clays, other algin, other celluloses, gums, and mixtures thereof.

Lubricants that can be used in pharmaceutical compositions and dosage forms include, but are not limited to, calcium stearate, magnesium stearate, mineral oil, light mineral oil, glycerin, sorbitol, mannitol, polyethylene glycol, other glycols, stearic acid, sodium lauryl sulfate,
15 talc, hydrogenated vegetable oil (*e.g.*, peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil, and soybean oil), zinc stearate, ethyl oleate, ethyl laureate, agar, and mixtures thereof. Additional lubricants include, for example, a syloid silica gel (AEROSIL200, manufactured by W.R. Grace Co. of Baltimore, MD), a coagulated aerosol of synthetic silica (marketed by Degussa Co. of Plano, TX), CAB-O-SIL (a pyrogenic silicon dioxide product sold
20 by Cabot Co. of Boston, MA), and mixtures thereof. If used at all, lubricants are typically used in an amount of less than about 1 weight percent of the pharmaceutical compositions or dosage forms into which they are incorporated.

In one embodiment, a solid oral dosage form of the invention comprises a compound provided herein, anhydrous lactose, microcrystalline cellulose, polyvinylpyrrolidone, stearic acid,
25 colloidal anhydrous silica, and gelatin.

5.8 Delayed Release Dosage Forms

Active ingredients may be administered by controlled release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; and
30 4,008,719, 5,674,533, 5,059,595, 5,591,767, 5,120,548, 5,073,543, 5,639,476, 5,354,556, and 5,733,566, each of which is incorporated herein by reference. Such dosage forms can be used to

provide slow or controlled-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Suitable controlled-release formulations known to those of ordinary skill in the art, including those described herein, can be readily selected for use with the active ingredients provided herein. Thus, provided herein are single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled-release.

All controlled-release pharmaceutical products have a common goal of improving drug therapy over that achieved by their non-controlled counterparts. Ideally, the use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include extended activity of the drug, reduced dosage frequency, and increased patient compliance. In addition, controlled-release formulations can be used to affect the time of onset of action or other characteristics, such as blood levels of the drug, and can thus affect the occurrence of side (*e.g.*, adverse) effects.

Most controlled-release formulations are designed to initially release an amount of drug (active ingredient) that promptly produces the desired therapeutic effect, and gradually and continually release of other amounts of drug to maintain this level of therapeutic or prophylactic effect over an extended period of time. In order to maintain this constant level of drug in the body, the drug must be released from the dosage form at a rate that will replace the amount of drug being metabolized and excreted from the body. Controlled-release of an active ingredient can be stimulated by various conditions including, but not limited to, pH, temperature, enzymes, water, or other physiological conditions or compounds.

5.9 Parenteral Dosage Forms

Parenteral dosage forms can be administered to patients by various routes including, but not limited to, subcutaneous, intravenous (including bolus injection), intramuscular, and intraarterial. Because their administration typically bypasses patients' natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited

to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions.

Suitable vehicles that can be used to provide parenteral dosage forms are well known to those skilled in the art. Examples include, but are not limited to: Water for Injection USP; aqueous vehicles such as, but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Compounds that increase the solubility of one or more of the active ingredients disclosed herein can also be incorporated into the parenteral dosage forms provided herein. For example, cyclodextrin and its derivatives can be used to increase the solubility of a compound and its derivatives. *See, e.g.*, U.S. Patent No. 5,134,127, which is incorporated herein by reference.

5.10 Topical and Mucosal Dosage Forms

Topical and mucosal dosage forms provided herein include, but are not limited to, sprays, aerosols, solutions, emulsions, suspensions, eye drops or other ophthalmic preparations, or other forms known to one of skill in the art. *See, e.g., Remington's Pharmaceutical Sciences*, 16th and 18th eds., Mack Publishing, Easton PA (1980 & 1990); and *Introduction to Pharmaceutical Dosage Forms*, 4th ed., Lea & Febiger, Philadelphia (1985). Dosage forms suitable for treating mucosal tissues within the oral cavity can be formulated as mouthwashes or as oral gels.

Suitable excipients (*e.g.*, carriers and diluents) and other materials that can be used to provide topical and mucosal dosage forms are well known to those skilled in the pharmaceutical arts, and depend on the particular tissue to which a given pharmaceutical composition or dosage form will be applied. With that fact in mind, typical excipients include, but are not limited to, water, acetone, ethanol, ethylene glycol, propylene glycol, butane-1,3-diol, isopropyl myristate, isopropyl palmitate, mineral oil, and mixtures thereof to form solutions, emulsions or gels, which are non-toxic and pharmaceutically acceptable. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms if desired. Examples of such additional ingredients are well known in the art. *See, e.g., Remington's Pharmaceutical Sciences*, 16th and 18th eds., Mack Publishing, Easton PA (1980 & 1990).

The pH of a pharmaceutical composition or dosage form may also be adjusted to improve delivery of one or more active ingredients. Similarly, the polarity of a solvent carrier, its ionic strength, or tonicity can be adjusted to improve delivery. Compounds such as stearates can also be added to pharmaceutical compositions or dosage forms to advantageously alter the hydrophilicity or lipophilicity of one or more active ingredients so as to improve delivery. In this regard, stearates can serve as a lipid vehicle for the formulation, as an emulsifying agent or surfactant, and as a delivery-enhancing or penetration-enhancing agent. Different salts, hydrates or solvates of the active ingredients can be used to further adjust the properties of the resulting composition.

5.11 Kits

In some embodiments provided herein, active ingredients are preferably not administered to a patient at the same time or by the same route of administration. Thus, provided herein are kits which, when used by the medical practitioner, can simplify the administration of appropriate amounts of active ingredients to a patient.

In one embodiment a kit provided herein comprises a compound provided herein, or a pharmaceutically acceptable salt, solvate or hydrate thereof. Kits may further comprise additional active agents, including but not limited to those disclosed herein.

Kits provided herein may further comprise devices that are used to administer the active ingredients. Examples of such devices include, but are not limited to, syringes, drip bags, patches, and inhalers.

Kits may further comprise cells or blood for transplantation as well as pharmaceutically acceptable vehicles that can be used to administer one or more active ingredients. For example, if an active ingredient is provided in a solid form that must be reconstituted for parenteral administration, the kit can comprise a sealed container of a suitable vehicle in which the active ingredient can be dissolved to form a particulate-free sterile solution that is suitable for parenteral administration. Examples of pharmaceutically acceptable vehicles include, but are not limited to: Water for Injection USP; aqueous vehicles such as, but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such as,

but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

5.12 Antibodies

Antibodies that immunospecifically bind to CRBN (anti-CRBN antibodies) provided
5 herein include, but are not limited to, synthetic antibodies, monoclonal antibodies, recombinantly produced antibodies, multispecific antibodies (including bi-specific antibodies), human antibodies, humanized antibodies, chimeric antibodies, intrabodies, single-chain Fvs (scFv) (*e.g.*, including monospecific, bispecific, *etc.*), camelized antibodies, Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv), anti-idiotypic (anti-Id) antibodies, and antigen- or epitope-binding
10 fragments of any of the above.

In particular, antibodies provided herein include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds to a CRBN antigen. The immunoglobulin molecules provided herein can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*,
15 IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. In a specific embodiment, an antibody provided herein is an IgG antibody, and, in certain embodiments, an IgG1 or IgG4.

Also provided herein is an isolated CRBN antibody, for example, "CRBN70," as prepared according to Example 6.20 or 6.21 below. In one embodiment, the antibody is a
20 polyclonal antibody. In another embodiment, the antibody is a monoclonal antibody. In some embodiments, the antibody is a rabbit polyclonal antibody. In other embodiments, the antibody is a rabbit monoclonal antibody.

In another embodiment, provided herein is an isolated antibody which immunospecifically binds to the epitope having an amino acid sequence EEFHGRTLHDDDC
25 (SEQ ID:1). In another embodiment, the antibody immunospecifically binds to the epitope having an amino acid sequence EEFHGRTLHDDDC (SEQ ID:1), wherein the peptide is coupled to Keyhole Limpet Hemocyanin (KLH). In one embodiment, the antibody is a polyclonal antibody. In another embodiment, the antibody is a monoclonal antibody. In some embodiments, the antibody is a rabbit polyclonal antibody. In other embodiments, the antibody
30 is a rabbit monoclonal antibody. In certain embodiments, the antibody immunospecifically binds peptide 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12).

In certain embodiments, provided herein is an antibody that immunospecifically binds CRBN and comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:5, or the VH domain, VH CDR1, VH CDR2, and/or VH CDR3 thereof. In other embodiment, the antibody immunospecifically binds CRBN and comprises a light chain having the amino acid sequence depicted in SEQ ID NO:7, or the VL domain, VL CDR1, VL CDR2, and/or VL CDR3 thereof. In some embodiments, the antibody comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:5, or the VH domain, VH CDR1, VH CDR2, and/or VH CDR3 thereof; and a light chain having the amino acid sequence depicted in SEQ ID NO:7, or the VL domain, VL CDR1, VL CDR2, and/or VL CDR3 thereof. In certain embodiments, the antibody immunospecifically binds CRBN and comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:9, or the VH domain, VH CDR1, VH CDR2, and/or VH CDR3 thereof. In other embodiment, the antibody immunospecifically binds CRBN and comprises a light chain having the amino acid sequence depicted in SEQ ID NO:11, or the VL domain, VL CDR1, VL CDR2, and/or VL CDR3 thereof. In some embodiments, the antibody comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:9, or the VH domain, VH CDR1, VH CDR2, and/or VH CDR3 thereof; and a light chain having the amino acid sequence depicted in SEQ ID NO:11, or the VL domain, VL CDR1, VL CDR2, and/or VL CDR3 thereof. In certain embodiments, the antibody immunospecifically binds peptide 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12).

Any of the CRBN antibodies provided herein can be used in any of the methods provided herein

Variants and derivatives of antibodies include antibody fragments that retain the ability to specifically bind to an epitope. Exemplary fragments include Fab fragments (an antibody fragment that contains the antigen-binding domain and comprises a light chain and part of a heavy chain bridged by a disulfide bond); Fab' (an antibody fragment containing a single antigen-binding domain comprising an Fab and an additional portion of the heavy chain through the hinge region); F(ab')₂ (two Fab' molecules joined by interchain disulfide bonds in the hinge regions of the heavy chains; the Fab' molecules may be directed toward the same or different epitopes); a bispecific Fab (a Fab molecule having two antigen binding domains, each of which may be directed to a different epitope); a single chain Fab chain comprising a variable region, also known as, a sFv (the variable, antigen-binding determinative region of a single light and heavy chain of an antibody linked together by a chain of 10-25 amino acids); a disulfide-linked

Fv, or dsFv (the variable, antigen-binding determinative region of a single light and heavy chain of an antibody linked together by a disulfide bond); a camelized VH (the variable, antigen-binding determinative region of a single heavy chain of an antibody in which some amino acids at the VH interface are those found in the heavy chain of naturally occurring camel antibodies); a
5 bispecific sFv (a sFv or a dsFv molecule having two antigen-binding domains, each of which may be directed to a different epitope); a diabody (a dimerized sFv formed when the VH domain of a first sFv assembles with the VL domain of a second sFv and the VL domain of the first sFv assembles with the VH domain of the second sFv; the two antigen-binding regions of the
10 diabody may be directed towards the same or different epitopes); and a triabody (a trimerized sFv, formed in a manner similar to a diabody, but in which three antigen-binding domains are created in a single complex; the three antigen binding domains may be directed towards the same or different epitopes). Derivatives of antibodies also include one or more CDR sequences of an antibody combining site. The CDR sequences may be linked together on a scaffold when two or more CDR sequences are present. In certain embodiments, the anti-CRBN antibody comprises a
15 single-chain Fv ("scFv"). scFvs are antibody fragments comprising the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFvs see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds.
20 Springer-Verlag, New York, pp. 269-315 (1994).

Provided herein are antibodies that immunospecifically bind to a CRBN epitope, the antibodies comprising derivatives of the VH and VL chains described herein that immunospecifically bind to a CRBN antigen or a CRBN epitope.. Standard techniques known to those of skill in the art can be used to introduce mutations in the nucleotide sequence encoding a
25 molecule of the invention, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis which results in amino acid substitutions. Preferably, the derivatives include less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid
30 substitutions relative to the original molecule. In a preferred embodiment, the derivatives have conservative amino acid substitutions are made at one or more predicted non-essential amino

acid residues. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

In another embodiment, an antibody that immunospecifically binds to a CRBN epitope comprises an amino acid sequence that is at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of CGN-6-1-11 OR CGN-6-1-11, or an antigen-binding fragment thereof, such as a VH domain, VL domain, VH chain, or VL chain. In one embodiment, an antibody that immunospecifically binds to a CRBN epitope comprises an amino acid sequence that is at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to an amino acid sequence depicted in SEQ ID NOS:5, 7, 9 or 11.

In a specific embodiment, an antibody that immunospecifically binds a CRBN antigen comprises an amino acid sequence of a VH chain and/or an amino acid sequence a VL chain encoded by a nucleotide sequence that hybridizes to (1) the complement of a nucleotide sequence encoding any one of the VH and/or VL chains depicted in SEQ ID NOS:5 or 9 (H chain) and/or SEQ ID NOS:7 or 11 (L chain) under stringent conditions (*e.g.*, hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45° C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65° C) under highly stringent conditions (*e.g.*, hybridization to filter-bound nucleic acid in 6xSSC at about 45° C followed by one or more washes in 0.1xSSC/0.2% SDS at about 68° C), or under other stringent hybridization conditions

which are known to those of skill in the art (see, for example, Ausubel, F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

The anti-CRBN antibodies may be from any animal origin including birds and mammals
5 (*e.g.*, human, murine, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken). In certain embodiments, the anti-CRBN antibodies are human or humanized monoclonal antibodies. As used herein, “human” antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from mice that express antibodies from human genes.

10 In certain embodiments, the anti-CRBN antibodies are fully human antibodies, such as fully human antibodies that immunospecifically bind a CRBN polypeptide, a CRBN polypeptide fragment, or a CRBN epitope. Such fully human antibodies would be advantageous over fully mouse (or other full or partial non-human species antibodies), humanized antibodies, or chimeric antibodies to minimize the development of unwanted or unneeded side effects, such as immune
15 responses directed toward non-fully human antibodies (*e.g.*, anti-CRBN antibodies derived from other species) when administered to the subject.

Anti-CRBN antibodies provided herein may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a CRBN polypeptide or may be specific for both a CRBN polypeptide as well as for a heterologous
20 epitope, such as a heterologous polypeptide or solid support material. In certain embodiments, the antibodies provided herein are monospecific for a given epitope of a CRBN polypeptide and do not immunospecifically bind to other epitopes.

In certain embodiments, provided herein are anti-CRBN antibodies that immunospecifically bind to a CRBN epitope (*e.g.*, EEFHGRTLHDDD (SEQ ID NO:1) or peptide 65-76 of human CRBN
25 (SEQ ID NO:12)) or a CRBN antigen, as well as methods of use thereof.

Standard techniques known to those of skill in the art can be used to introduce mutations in the nucleotide sequence encoding an anti-CRBN provided herein, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis which results in amino acid substitutions. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with
30 an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include

amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*,
5 threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

10 In some embodiments, the antibody is a fully human anti-human CRBN antibody, such as a fully human monoclonal antibody. Fully human antibodies may be produced by any method known in the art. Exemplary methods include immunization with a CRBN antigen (any CRBN polypeptide capable of eliciting an immune response, and optionally conjugated to a carrier) of transgenic animals (*e.g.*, mice) that are capable of producing a repertoire of human antibodies in
15 the absence of endogenous immunoglobulin production; *see, e.g.*, Jakobovits *et al.*, (1993) *Proc. Natl. Acad. Sci.*, 90:2551; Jakobovits *et al.*, (1993) *Nature*, 362:255-258 (1993); Bruggermann *et al.*, (1993) *Year in Immunol.*, 7:33. Other methods of producing anti-CRBN antibodies can be found in the Examples provided herein.

Alternatively, fully human antibodies may be generated through the *in vitro* screening of phage
20 display antibody libraries; *see e.g.*, Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991), incorporated herein by reference. Various antibody-containing phage display libraries have been described and may be readily prepared by one skilled in the art. Libraries may contain a diversity of human antibody sequences, such as human Fab, Fv, and scFv fragments, that may be screened against an appropriate target.

25 The anti-CRBN antibodies include antibodies that are chemically modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody. For example, but not by way of limitation, the antibody derivatives include antibodies that have been chemically modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.*
30 Any of numerous chemical modifications may be carried out by known techniques, including,

but not limited to specific chemical cleavage, acetylation, formulation, metabolic synthesis of tunicamycin, *etc.* Additionally, the antibody may contain one or more non-classical amino acids.

In certain embodiments, anti-CRBN antibodies that immunospecifically bind to a CRBN antigen comprise a framework region known to those of skill in the art (*e.g.*, a human or non-human fragment). The framework region may, for example, be naturally occurring or consensus framework regions. In some embodiments, the framework region of an anti-CRBN antibody is human (see, *e.g.*, Chothia *et al.*, 1998, *J. Mol. Biol.* 278:457-479 for a listing of human framework regions, which is incorporated by reference herein in its entirety). See also Kabat *et al.* (1991) Sequences of Proteins of Immunological Interest (U.S. Department of Health and Human Services, Washington, D.C.) 5th ed.

In certain embodiments, the anti-CRBN antibodies provided herein are chimeric or humanized antibodies. In some embodiments, the antibodies provided herein comprise human framework regions with one or more amino acid substitutions at one, two, three or more of the following residues: (a) rare framework residues that differ between the murine antibody framework (*i.e.*, donor antibody framework) and the human antibody framework (*i.e.*, acceptor antibody framework); (b) Venier zone residues when differing between donor antibody framework and acceptor antibody framework; (c) interchain packing residues at the VH/VL interface that differ between the donor antibody framework and the acceptor antibody framework; (d) canonical residues which differ between the donor antibody framework and the acceptor antibody framework sequences, particularly the framework regions crucial for the definition of the canonical class of the murine antibody CDR loops; (e) residues that are adjacent to a CDR; (g) residues capable of interacting with the antigen; (h) residues capable of interacting with the CDR; and (i) contact residues between the VH domain and the VL domain. In certain embodiments, antibodies that immunospecifically bind to a CRBN antigen comprising the human framework regions with one or more amino acid substitutions at one, two, three or more of the above-identified residues are antagonistic CRBN antibodies.

In other embodiments, fusion proteins comprising an anti-CRBN antibody are provided herein that immunospecifically binds to a CRBN antigen and a heterologous polypeptide.

5.13 Diagnostic Use of Antibodies

Labeled CRBN antibodies provided herein and derivatives and analogs thereof, which immunospecifically bind to a CRBN antigen can be used for diagnostic purposes to detect, diagnose, or monitor CRBN expression levels or a CRBN-mediated disease in a patient.

In some embodiments, provided herein are methods of utilizing a CRBN antibody to
5 measure expression levels of CRBN in patient tumor or host cells, to predict clinical response to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof. Also provided herein are methods of utilizing a CRBN antibody provided herein to measure expression levels of CRBN in
10 patient tumor or host cells, to monitor clinical response to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof. Also provided herein are methods of utilizing a CRBN antibody provided herein to measure expression levels of CRBN in patient tumor or host cells, to monitor
15 patient compliance to dosing to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof. Also provided herein are methods of utilizing a CRBN antibody provided herein to measure expression levels of CRBN in patient tumor or host cells, to monitor development of resistance
20 to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

Provided herein are diagnostic assays for diagnosing a CRBN-mediated disease comprising: (a) assaying for the level of a CRBN antigen in cells or a tissue sample of an
25 individual using one or more antibodies of the invention that immunospecifically bind to a CRBN antigen; and (b) comparing the level of the CRBN antigen with a control level, *e.g.*, levels in normal tissue samples, whereby an increase in the assayed CRBN antigen level compared to the control level of the CRBN antigen is indicative of a CRBN-mediated disease.

Antibodies provided herein can be used to assay CRBN antigen levels in a biological
30 sample using classical immunohistological methods as described herein or as known to those of skill in the art (*e.g.*, see Jalkanen *et al.*, 1985, J. Cell. Biol. 101:976-985; and Jalkanen *et al.*,

1987, J. Cell . Biol. 105:3087-3096). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (^{125}I , ^{121}I),
5 carbon (^{14}C), sulfur (^{35}S), tritium (^3H), indium (^{121}In), and technetium (^{99}Tc); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Provided herein are methods for the detection of CRBN in a patient. In one embodiment, the method comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled antibody that immunospecifically
10 binds to a CRBN antigen; b) waiting for a time interval following the administering for permitting the labeled antibody to preferentially concentrate at sites in the subject where the CRBN antigen is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled antibody in the subject, such that detection of labeled antibody above the background level indicates that the subject has
15 increased CRBN expression. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case
20 of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of ^{99}Tc . The labeled antibody will then preferentially accumulate at the location of cells which contain the specific protein. *In vivo* tumor imaging is described in S.W. Burchiel *et al.*, "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer,
25 S.W. Burchiel and B.A. Rhodes, eds., Masson Publishing Inc. (1982).

Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled antibody to preferentially concentrate at sites in the subject and for unbound labeled antibody to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment
30 the time interval following administration is 5 to 20 days or 5 to 10 days.

Presence of the labeled molecule can be detected in the subject using methods known in the art for *in vivo* scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston *et al.*, U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patient using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

6. EXAMPLES

Certain embodiments of the invention are illustrated by the following non-limiting examples.

6.1 Preparation of 3-(4-amino-1-oxo-1,3-dihydro-isoindol-2-yl)-piperidine-2,6-dione (lenalidomide)

Methyl 2-bromomethyl-3-nitrobenzoate

A stirred mixture of methyl 2-methyl-3-nitrobenzoate (14.0 g, 71.7 mmol) and N-bromosuccinimide (15.3 g, 86.1 mmol) in carbon tetrachloride (200 mL) was heated under gentle reflux for 15 hours while a 100W bulb situated 2 cm away was shining on the flask. The mixture was filtered and the solid was washed with methylene chloride (50 mL). The filtrate was washed with water (2x100 mL), brine (100 mL) and dried. The solvent was removed in vacuo and the residue was purified by flash chromatography (hexane/ethyl acetate, 8/2) to afford 19 g (96%) of the product as a yellow solid: mp 70.0-71.5°C; ¹H NMR (CDCl₃) δ 8.12-8.09(dd, J=1.3 and 7.8 Hz, 1H), 7.97-7.94(dd, J=1.3 and 8.2 Hz, 1H), 7.54(t, J=8.0 Hz, 1H), 5.15(s, 2H), 4.00(s, 3H); ¹³C NMR (CDCl₃) δ 165.85, 150.58, 134.68, 132.38, 129.08, 127.80, 53.06, 22.69; HPLC, Water Nove-Pak/C18, 3.9x150 mm, 4 micron, 1mL/min, 240 nm, 40/60 CH₃CN/0.1%H₃PO₄(aq) 7.27 min(98.92%); Anal. Calcd for C₉H₈NO₄Br : C, 39.44; H, 2.94; N, 5.11; Br, 29.15. Found : C, 39.46; H, 3.00; N, 5.00; Br, 29.11.

t-Butyl N-(1-oxo-4-nitroisindolin-2-yl)-L-glutamine

Triethylamine (2.9 g, 28.6 mmol) was added dropwise to a stirred mixture of methyl 2-bromomethyl-3-nitrobenzoate (3.5 g, 13.0 mmol) and L-glutamine t-butyl ester hydrochloride (3.1 g, 13.0 mmol) in tetrahydrofuran (90 mL). The mixture was heated to reflux for 24 hours.

5 To the cooled mixture was added methylene chloride (150 mL) and the mixture was washed with water (2 x 40 mL), brine (40 mL) and dried. The solvent was removed in vacuo and the residue was purified by flash chromatography (3% CH₃OH in methylene chloride) to afford 2.84 g (60%) of crude product which was used directly in the next reaction: ¹H NMR (CDCl₃) δ 8.40(d, J=8.1 Hz, 1H), 8.15(d, J=7.5 Hz, 1H), 7.71(t, J=7.8 Hz, 1H), 5.83(s, 1H), 5.61(s, 1H), 5.12(d, J=19.4 Hz, 1H), 5.04-4.98(m, 1H), 4.92(d, J=19.4 Hz, 1H), 2.49-2.22(m, 4H). 1.46(s, 9H); HPLC, Waters Nova-Pak C18, 3.9x150 mm, 4 micron, 1 mL/min, 240 nm, 25/75 CH₃CN/0.1%H₃PO₄(aq) 6.75 min(99.94%).

N-(1-oxo-4-nitroisindolin-2-yl)-L-glutamine

Hydrogen chloride gas was bubbled into a stirred 5°C solution of t-butyl N-(1-oxo-4-nitroisindolin-2-yl)-L-glutamine (3.6 g, 9.9 mmol) in methylene chloride (60 mL) for 1 hour. The mixture was then stirred at room temperature for another hour. Ether (40 mL) was added and the resulting mixture was stirred for 30 minutes. The slurry was filtered, washed with ether and dried to afford 3.3 g of the product: ¹H NMR (DMSO-d₆) δ 8.45(d, J=8.1 Hz, 1H), 8.15(d, J=7.5 Hz, 1H), 7.83(t, J=7.9 Hz, 1H), 7.24(s, 1H), 6.76(s, 1H), 4.93(s, 2H), 4.84-4.78(dd, J=4.8 and 10.4 Hz, 1H), 2.34-2.10(m, 4H); ¹³C NMR (DMSO-d₆) δ 173.03, 171.88, 165.96, 143.35, 137.49, 134.77, 130.10, 129.61, 126.95, 53.65, 48.13, 31.50, 24.69; Anal. Calcd for C₁₃H₁₃N₃O₆ : C, 50.82; H, 4.26; N, 13.68. Found : C, 50.53; H, 4.37; N, 13.22.

(S)-3-(1-oxo-4-nitroisindolin-2-yl)piperidine-2,6-dione

A stirred suspension mixture of N-(1-oxo-4-nitroisindolin-2-yl)-L-glutamine (3.2 g, 10.5 mmol) in anhydrous methylene chloride (150 mL) was cooled to -40°C with isopropanol/dry ice bath. Thionyl chloride (0.82 mL, 11.3 mmol) was added dropwise to the cooled mixture followed by pyridine (0.9 g, 11.3 mmol). After 30 min, triethylamine (1.2 g, 11.5 mmol) was added and the mixture was stirred at -30 to -40°C for 3 hours. The mixture was poured into ice water (200 mL) and the aqueous layer was extracted with methylene chloride (40 mL). The methylene chloride solution was washed with water (2 x 60 mL), brine (60 mL) and dried. The solvent was removed in vacuo and the solid residue was slurried with ethyl acetate (20 mL) to give 2.2 g (75%) of the

product as a white solid: mp 285°C; ¹H NMR (DMSO-d₆) δ : 1.04(s, 1H), 8.49-8.45(dd, J=0.8 and 8.2 Hz, 1H), 8.21-8.17(dd, J=7.3 Hz, 1H), 7.84(t, J=7.6 Hz, 1H), 5.23-5.15(dd, J=4.9 and 13.0 Hz, 1H), 4.96(dd, J=19.3 and 32.4 Hz, 2H), 3.00-2.85(m, 1H), 2.64-2.49(m, 2H), 2.08-1.98(m, 1H); ¹³C NMR (DMSO- d₆) δ 172.79, 170.69, 165.93, 143.33, 137.40, 134.68, 130.15, 129.60, 127.02, 51.82, 48.43, 31.16. 22.23; HPLC, Waters Nove-Pak/C18, 3.9x150 mm, 4 micron, 1 mL/min, 240 nm, 20/80 CH₃CN/0.1%H₃PO₄(aq) 3.67 min(100%); Anal. Calcd for C₁₃H₉N₃O₅ : C, 53.98; H, 3.83; N, 14.53. Found : C, 53.92; H, 3.70; N, 14.10.

3-(4-amino-1-oxo-1,3-dihydro-isoindol-2-yl)-piperidine-2,6-dione

A mixture of (S)-3-(1-oxo-4-nitroisoindolin-2-yl)piperidine-2,6-dione (1.0 g, 3.5 mmol) and 10% Pd/C (0.3 g) in methanol (600 mL) was hydrogenated in a Parr-Shaker apparatus at 50 psi of hydrogen for 5 hours. The mixture was filtered through Celite and the filtrate was concentrated in vacuo. The solid was slurried in hot ethyl acetate for 30 min, filtered and dried to afford 0.46 g (51%) of the product as a white solid: mp 235.5-239°C; ¹H NMR (DMSO-d₆) δ 11.01 (s, 1H). 7.19(t, J=7.6 Hz, 1H). 6.90(d, J=7.3 Hz, 1H), 6.78(d, J=7.8 Hz, 1H), 5.42(s, 2H). 5.12(dd, J=5.1 and 13.1 Hz, 1H), 4.17(dd, J=17.0 and 28.8 Hz, 2H), 2.92-2.85(m, 1H). 2.64-2.49(m, 1H). 2.34-2.27(m, 1H), 2.06-1.99(m, 1H); ¹³C NMR (DMSO-d₆) δ 172.85, 171.19, 168.84, 143.58, 132.22. 128.79, 125.56, 116.37, 110.39, 51.48, 45.49, 31.20, 22.74; HPLC. Waters Nova-Pak/C18, 3.9x150 mm, 4 micron, 1 mL/min, 240 nm, 10/90 CH₃CN/0.1%H₃PO₄(aq) 0.96 min(100%); Chiral analysis, Daicel Chiral Pak AD, 40/60 Hexane/IPA, 6.60 min(99.42%); Anal. Calcd for C₁₃H₁₃N₃O₃ : C, 60.23; H, 5.05; N, 16.21. Found : C, 59.96; H, 4.98; N, 15.84.

3-(4-Amino-1-oxo-1,3-dihydro-isoindol-2-yl)-piperidine-2,6-dione may also be prepared by methods known in the art, for example, as provided in *Drugs of the Future*, 2003, 28(5): 425-431, the entirety of which is incorporated by reference.

6.2 Preparation of 4-amino-2-(2,6-dioxopiperidin-3-yl)-1H-isoindole-1,3-dione (pomalidomide)

The preparation of 4-amino-2-(2,6-dioxopiperidin-3-yl)-1H-isoindole-1,3-dione is described, for example, in U.S. patent nos. 7,812,169 and 7,709,502, the entirety of each of which is incorporated by reference.

Into a stirring solution of carboxybenzyloxy-L-glutamine (2.8 g, 10 mmols) in 40 mL anhydrous THF, 1,1-carbonyldiimidazole (1.92 g, 12 mmols) were added. The reaction mixture was heated under reflux for 18 hours. The THF was evaporated and the product was dissolved in

chloroform. The chloroform layer was washed with water and brine and dried over anhydrous CaSO_4 , filtered and evaporated to give white solid. The solid product was crystallized from ethyl ether to give 2.4 grams crystalline powder (90%). (Alternatively, carboxybenzyloxy-L-glutamine can be cyclized by treating with SOCl_2 in N,N-dimethylformamide at -70°C to 0°C for 1 hour to form the product). The reaction mixture was diluted with CHCl_3 and washed with 5% Na_2CO_3 , dried over anhydrous Na_2SO_4 , filtered, and evaporated to give 2.5 g (90% yield) S(-)-(3-benzyloxycarbonylamino)-glutarimide). ^1H NMR (CDCl_3) δ 8.2 (1H, s broad), 7.4 (5H, s, aromatic), 5.8 (1H, d), 5.15 (2H, s), 4.4 (1H, dd, $J=4.5, 3$), 2.95-2.4 (3H, m), 1.86 (1H, d, t, $J=11.5, 6.5$). m.p. $122-124^\circ\text{C}$ (lit. $122-124^\circ\text{C}$).

Into a solution of S(-)-(2-benzyloxycarbonylamino)glutarimide (1.2 g, 4.6 mmols) in 15 mL acetic acid glacial, 8 mL of 30% HBr/ acetic acid solution was added at 20°C . The temperature of reaction mixture was raised to RT and stirred for 1 hour. White solid powder of S(-)-2-amino-glutarimide HBr started appearing in reaction mixture. The solid was filtered and washed with 5 mL acetic acid glacial and then with ether to give 1.8 g (80%) product. Analysis on polarimeter of product showed (-) rotation, $[\alpha]^{25}_{\text{D}}$ ($c=1$, water) = -37.5° and confirmed the product as S(-)-2-amino-glutarimide. ^1H NMR in $\text{DMSO}-\text{D}_6$ confirmed the product as 2-amino-L-glutarimide HBr.

Into a solution of (4.18 g, 20 mmols S(-)-2-amino-glutarimide HBr in 50 mL of anhydrous DMF, 3.8 g (20 mmols) of 3-nitrophthalic anhydride was added. After adding 100 mL acetic acid (glacial), the reaction mixture was heated at about 70°C to about 80°C for about 24 hours. Thereafter, the solvents were evaporated under vacuum to yield an off-white solid. On adding 10 mL ethyl alcohol to the solid, an off-white powder product was formed. The product was separated and washed with 20 mL ethyl alcohol. ^1H NMR ($\text{DMSO}-\text{D}_6$) δ 11.25 (1H, s broad), 8.35 (1H, d, $J=7.2$), 8.25 (1H, d, $J=7.0$), 8.15 (1H, t, $J=8.0$), 5.2 (1H, dd, $J=5.5, 7.2$), 3.00-2.85 (1H, m), 2.65-2.4 (2H, m), 2.15-2.05 (1H, m). m.p.: $228-229^\circ\text{C}$ (lit. $228.5-229.5^\circ\text{C}$).

4-Nitro-thalidomide (1 g, 3.3 mmols) was dissolved in 50 mL dioxane/methanol 4:1 mixture and hydrogenated in a Parr hydrogenator at 40 psi of hydrogen in the presence of a Pd/C 5% catalyst for about 4 hours. After filtering the reaction mixture through a Celite filtering agent, the solvents were evaporated under vacuum to yield a yellow powder. The product was recrystallized from ethyl acetate/dioxane to yield 800 mg (85% purity) of S(-)-4-amino-thalidomide. ^1H NMR in $\text{DMSO}-\text{D}_6$: 11.10 (1H, s broad), 7.45 (1H, t, $J=7.5$), 7.05 (1H, d, $J=5.2$),

6.95 (1H, d, $J=5.2$), 6.5 (2H, s broad), 5.05 (1H, dd, $J=5.0$, 13.42), 2.95-2.80 (1H, m), 2.65-2.5 (2H, m), 2.05-1.95 (1H, m). m.p. 318.2-319.5°C. Absolute configuration was determined by comparison of specific rotation $[\alpha]^{25}_D$ of (R)- and (S)-4-amino-2-(2,6-dioxopiperidin-3-yl)-1H-isoindole-1,3-dione to the analogous compounds R(+)- and S(-)-thalidomide. Analysis on
5 polarimeter of product showed (-) rotation, $[\alpha]^{25}_D$ (C=0.5, dioxane) = -27.70° and confirmed the product as S(-)-4-amino-2-(2,6-dioxopiperidin-3-yl)-1H-isoindole-1,3-dione.

The two enantiomers were resolved by chiral HPLC column Welk-01 (10 mm x 750 mm) and eluted with CH₃CN/MeOH/H₂O 1:1:5 mixture. The retention time for the S(-) enantiomer was 33.74 minutes and for the R(+) enantiomer 35.62 minutes at a flow rate of 2 mL/min at 240
10 nm, respectively.

6.3 Preparation of 3-(5-amino-2-methyl-4-oxo-4H-quinazolin-3-yl)-piperidine-2,6-dione (Compound B)

To a solution of potassium hydroxide (16.1 g, 286 mmol) in water (500 mL), was added 3-nitrophthalimide (25.0 g, 130 mmol) in portion at 0 °C. The suspension was stirred at 0 °C for
15 3 hrs, and then heated to 30 °C for 3 hrs. To the solution, was added HCl (100 mL, 6N). The resulting suspension was cooled to 0 °C for 1 hr. The suspension was filtered and washed with cold water (2 x 10 mL) to give 3-nitro-phthalamic acid as a white solid (24.6 g, 90% yield): ¹H NMR (DMSO-d₆) δ 7.69 (brs, 1H, NHH), 7.74 (t, $J=8$ Hz, 1H, Ar), 7.92 (dd, $J=1, 8$ Hz, 1H, Ar), 8.13 (dd, $J=1, 8$ Hz, 1H, Ar), 8.15 (brs, 1H, NHH), 13.59 (s, 1H, OH); ¹³C NMR (DMSO-
20 d₆) δ 125.33, 129.15, 130.25, 132.54, 136.72, 147.03, 165.90, 167.31.

To a mixture of 3-nitro-phthalamic acid (24.6 g, 117 mmol) and potassium hydroxide (6.56 g, 117 mmol) in water (118 mL), was added a mixture of bromine (6 mL), potassium hydroxide (13.2 g, 234 mmol) in water (240 mL) at 0 °C, followed by addition of a solution of potassium hydroxide (19.8 g, 351 mmol) in water (350 mL). After 5 minutes at 0 °C, the
25 mixture was heated in a 100 °C oil bath for 1 hr. The reaction solution was cooled to room temperature, and then, in an ice-water bath for 30 minutes. To the mixture, a solution of HCl (240 mL, 2N) was added dropwise at 0 °C, and the resulting mixture was kept for 1 hr. The suspension was filtered and washed with water (5 mL) to give 2-amino-6-nitro-benzoic acid as yellow solid (15.6 g, 73% yield): HPLC: Waters Symmetry C₁₈, 5 μ m, 3.9 x 150 mm, 1 mL/min, 240 nm, CH₃CN/0.1% H₃PO₄, 5% grad to 95% over 5 min, 5.83 min (85%); ¹H NMR (DMSO-
30 d₆) δ 6.90 (dd, $J=1, 8$ Hz, 1H, Ar), 7.01 (dd, $J=1, 9$ Hz, 1H, Ar), 7.31 (t, $J=8$ Hz, 1H, Ar),

8.5-9.5 (brs, 3H, OH, NH₂); ¹³C NMR (DMSO-d₆) δ 105.58, 110.14, 120.07, 131.74, 149.80, 151.36, 166.30; LCMS: MH = 183.

A mixture of 2-amino-6-nitro-benzoic acid (1.5 g, 8.2 mmol) in acetic anhydride (15 mL) was heated at 200 °C for 30 minutes in a microwave oven. The mixture was filtered and washed with ethyl acetate (20 mL). The filtrate was concentrated *in vacuo*. The solid was stirred in ether (20 mL) for 2 hrs. The suspension was filtered and washed with ether (20 mL) to give 2-methyl-5-nitro-benzo[d][1,3]oxazin-4-one as a light brown solid (1.4 g, 85% yield): HPLC: Waters Symmetry C₁₈, 5μm, 3.9 x 150 mm, 1 mL/min, 240 nm, CH₃CN/0.1% H₃PO₄, 5% grad 95% in 5 min, 5.36 min (92%); ¹H NMR (DMSO-d₆) δ 2.42 (s, 3H, CH₃), 7.79 (dd, *J* = 1, 8 Hz, 1H, Ar), 7.93 (dd, *J* = 1, 8 Hz, 1H, Ar), 8.06 (t, *J* = 8 Hz, 1H, Ar); ¹³C NMR (DMSO-d₆) δ 20.87, 107.79, 121.54, 128.87, 137.19, 147.12, 148.46, 155.18, 161.78; LCMS: MH = 207.

Two vials each with a suspension of 5-nitro-2-methyl-benzo[d][1,3]oxazin-4-one (0.60 g, 2.91 mmol) and 3-amino-piperidine-2,6-dione hydrogen chloride (0.48 g, 2.91 mmol) in pyridine (15 mL) were heated at 170 °C for 10 minutes in a microwave oven. The suspension was filtered and washed with pyridine (5 mL). The filtrate was concentrated *in vacuo*. The resulting mixture was stirred in HCl (30 mL, 1N), ethyl acetate (15 mL) and ether (15 mL) for 2 hrs. The suspension was filtered and washed with water (30 mL) and ethyl acetate (30 mL) to give a dark brown solid, which was stirred with methanol (50 mL) at room temperature overnight. The suspension was filtered and washed with methanol to give 3-(2-methyl-5-nitro-4-oxo-4H-quinazolin-3-yl)-piperidine-2,6-dione as a black solid (490 mg, 27% yield). The solid was used in the next step without further purification.

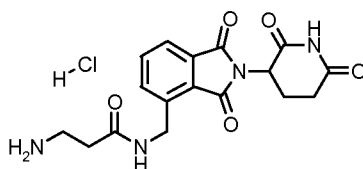
A mixture of 3-(2-methyl-5-nitro-4-oxo-4H-quinazolin-3-yl)-piperidine-2,6-dione (250 mg) and Pd(OH)₂ on carbon (110 mg) in DMF (40 mL) was shaken under hydrogen (50 psi) for 12 hrs. The suspension was filtered through a pad of Celite and washed with DMF (10 mL).

The filtrate was concentrated *in vacuo* and the resulting oil was purified by flash column chromatography (silica gel, methanol/methylene chloride) to give 3-(5-amino-2-methyl-4-oxo-4H-quinazolin-3-yl)-piperidine-2,6-dione as a white solid (156 mg, 69% yield): HPLC: Waters Symmetry C₁₈, 5μm, 3.9 x 150 mm, 1 mL/min, 240 nm, 10/90 CH₃CN/0.1% H₃PO₄, 3.52 min (99.9%); mp: 293-295 °C; ¹H NMR (DMSO-d₆) δ 2.10-2.17 (m, 1H, CHH), 2.53 (s, 3H, CH₃), 2.59-2.69 (m, 2H, CH₂), 2.76-2.89 (m, 1H, CHH), 5.14 (dd, *J* = 6, 11 Hz, 1H, NCH), 6.56 (d, *J* = 8 Hz, 1H, Ar), 6.59 (d, *J* = 8 Hz, 1H, Ar), 7.02 (s, 2H, NH₂), 7.36 (t, *J* = 8 Hz, 1H, Ar), 10.98 (s,

^1H , NH); ^{13}C NMR (DMSO-d_6) δ 20.98, 23.14, 30.52, 55.92, 104.15, 110.48, 111.37, 134.92, 148.17, 150.55, 153.62, 162.59, 169.65, 172.57; LCMS: $\text{MH} = 287$; Anal. Calcd. for $\text{C}_{14}\text{H}_{14}\text{N}_4\text{O}_3 + 0.3 \text{ H}_2\text{O}$: C, 57.65; H, 5.05; N, 19.21. Found: C, 57.50; H, 4.73; N, 19.00.

6.4 Identification of direct compound targets

5 To identify the direct target of thalidomide and other related drugs, we develop an affinity purification technique. A compound control ("Compound A") coupled to Affigel-10 (10 μmol drug per mg Affigel) was used for affinity purification experiments.



Compound A ($\text{TNF IC}_{50}=622 \text{ nM}$; Jurkat IL-2 $\text{EC}_{200}=3.47 \mu\text{M}$)

10 Proteins were isolated from Jurkat T cell lysates by binding to the Compound A-Affigel followed by elution using free Compound A. Coomassie stained bands were excised and sent to Harvard Microchemistry Facility for sequence analysis. The proteins were proteolytically digested and analyzed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer. The MS/MS
15 spectra were correlated with known sequences using the algorithm Sequest and other programs, then the peptide sequences were reviewed by a scientist for consensus with known proteins and the results manually confirmed for fidelity.

Summary of results: DDB1 (DNA damage binding protein 1 (XPCE, human) was affinity purified from Jurkat extracts using Compound A-immobilized beads. DDB1 was highly
20 represented in the eluted fractions (108 peptides) compare with for example with the second proteins most represented Glycogen branching enzyme (55 peptides). DDB1 directly interacts with CRBN and could had been pull-down by virtue of its binding to CRBN.

6.5 CRBN siRNAs knocked down CRBN in sensitive multiple myeloma cell lines

25 The role of cereblon (CRBN) in lenalidomide MOA was confirmed using knock-down experiments in two lenalidomide sensitive multiple myeloma cell lines, H929 and U266B1. It was found that down regulation of CRBN abrogates drug-induced cell cycle arrest, activation of

tumor suppressors, inhibition of oncogenes and global changes in gene expression profiles and ubiquitination.

10 different single and pool siRNAs against CRBN were evaluated in H929 and U266B1 cells. RT-PCR was used to test the knockdown efficiency after 24 and 48 hour transfection. The results showed that the CRBN-siRNA-1, CRBN-siRNA-7, CRBN-siRNA-9, CRBN-siRNA-10, and CRBN-siRNA-11 significantly reduced the expression of CRBN mRNA as compared with other siRNAs and mock siRNA (Table 1).

Table 1: Effect of CRBN-siRNAs on CRBN gene expression

siRNA	% inhibition of CRBN mRNA relative to CRBN mRNA levels in Mock control		
	siRNA conc		
	10 nM	25 nM	50 nM
Invitrogen siRNAs			
Low GC Neg ctrl	88 / 100	85.5 / 77.5	85 / 77
CRBN-7	22 / 25	14 / 17.5	16.5 / 17
CRBN-8	47.5 / 61	29.5 / 41	33 / 37.5
CRBN-9	67.5 / 66.4	61.5 / 52	42 / 37
Dharmacon siRNAs			
Smart pool neg ctrl	87 / 78	72.5 / 84	98.5 / 88.5
CRBN Smartpool	28 / 34.5	19.3 / 28.5	18 / 17
CRBN-9	55 / 40	28.5 / 33	17.5 / 23
CRBN-10	23 / 35	20.5 / 29	20 / 22.5
J-021086-11	22 / 27	20 / 17	16.3 / 15.3
J-021086-12	55 / 53	37 / 39	21 / 25

6.6 Knockdown of CRBN abrogates the anti-proliferative effect of drugs in multiple myeloma cells

Drugs such as lenalidomide and pomalidomide have direct anti-proliferative activity against MM cells by inducing cell cycle arrest in G1 phase, followed by a decrease in viability. To study the role of CRBN in lenalidomide and other drugs anti-proliferative activities, H929 and U266B1 cells, two sensitive myeloma lines, were transfected with CRBN-siRNAs or control siRNAs for 24, 48, 72 and 96 hours. Cells were treated 24 h after transfection with DMSO (0.1 %), pomalidomide (1 μ M) and lenalidomide (10 μ M) for 1, 2, 3 days to evaluated the

compound effect on CRBN protein, mRNA expression and the effect on cell cycle and proliferation. RT-PCR and western blot assays showed that CRBN siRNAs knocked down CRBN. CRBN down regulation was confirmed by RT-PCR and Western blot using the CRBN70 antibody (Figure 1). Treatment with lenalidomide and pomalidomide affected neither mRNA nor protein expression of CRBN significantly.

Lenalidomide and pomalidomide induced a delay of cell cycle progression, measured as the decrease of the number of cells in S phase, of 40 % and 50 % respectively in control mock and negative control siRNA-transfected cells (average inhibition of 3 days of treatment) (Figures 2A & 2B). Knockdown of CRBN markedly abrogated the lenalidomide and pomalidomide-induced delay in cell cycle progression in U266B1 cells. *Id.*

The effect of CRBN in H929 cells was also evaluated. H929 cells were transfected with mock, negative control siRNA and CRBN-siRNA-7 for 24, 48, 72 and 96 h. Cells were treated 24 h after transfection with DMSO (0.1 %), pomalidomide (1 μ M), lenalidomide (10 μ M) or 3-(5-amino-2-methyl-4-oxo-4H-quinazolin-3-yl)-piperidine-2,6-dione ("Compound B") for 1, 2, 3 days and the effect on cell cycle and proliferation investigated. Lenalidomide, pomalidomide and Compound B induced a delay of cell cycle progression, measured as the decrease of the number of cells in S phase, in control mock and negative control siRNA-transfected cells after 72 h treatment (Figure 2C). Knockdown of CRBN markedly abrogated immunomodulatory drugs-induced delay in cell cycle progression in H929 cells (Figure 2C) from of 50 to 4% for lenalidomide, from 70 to 15% for pomalidomide, and from 65 to 22% for Compound B.

6.7 Knockdown of CRBN abrogated effect of drugs on cell cycle, tumor suppressor and apoptotic proteins

To further investigate the effects of CRBN on the cell cycle arrest induced by drugs we used RT-PCR and Western blot analysis to measure the levels of key cell cycle and apoptotic regulators. U266B1 cells were transfected with mock, negative control siRNA, CRBN-siRNA-7 and CRBN-siRNA-11 for 24, 48, 72 and 96 h (Figure 3A). Cells were treated 24 h after transfection with DMSO (0.1 %), pomalidomide (1 μ M) and lenalidomide (10 μ M) for 1, 2, 3 days and the effect on mRNA and protein levels of the cyclin-dependent kinase (CDK) inhibitor p21^{WAF-1} were evaluated. It has been shown that lenalidomide- and pomalidomide-induced cell cycle arrest is dependent on up-regulation of p21^{WAF-1} in U2669. Our results showed that p21 was up-regulated by lenalidomide and pomalidomide in control mock and negative control

siRNA-transfected cells (Figure 3C) indicating a decrease of S phase cells resulted from lenalidomide and pomalidomide G1 arrest (Figure 3B). However, knockdown of CRBN prevents the induction of p21^{WAF-1} indicating the successful abrogation of G1 arrest and renewal of S phase progression (Figure 3C & 3D).

5 In H929 cells, the cell cycle arrest in G1 phase by the drugs coincides with a reduction of tumor suppressor, pRb, phosphorylation and the oncogene and myeloma survival factor IRF4. Western blot analysis showed that lenalidomide, pomalidomide and Compound B decreased phosphorylation of pRB (Figures 4A & 4B) and total level of protein IRF4 (Figures 4C & 4D). The effect of the drugs was reduced by knockdown of CRBN suggesting that inhibition of cell
10 cycle progression by the drugs requires CRBN protein.

6.8 Knockdown of CRBN inhibits lenalidomide and pomalidomide effects on gene expression

Gene expression profile using microarray technology identified 759 or 609 genes differentially modulated in negative control siRNA-transfected U266B1 cells treated with
15 pomalidomide (1 μ M) or lenalidomide (10 μ M) compared to DMSO treated control ($\pm$ 1.7 fold; $P < 0.05$ ANOVA p-value) (Figures 5A & 5B).

Representative significant Gene Ontology (GO) classes of down regulated genes in pomalidomide include: cell cycle (87 genes; $P = 5.2E-56$), mitosis (43 genes; $P = 1.2E-39$), cytoskeleton (58 genes; $P = 2.6E-16$) and response to DNA damage stimulus (36 genes; $P =$
20 $1.8E-21$). Representative significant Gene Ontology (GO) classes of up regulated genes include: antigen processing and presentation (12 genes; $P = 2.8E-17$), immune response (35 genes; $P = 5.70E-14$) and cell death (50 genes; $P = 1.7E-10$).

Representative significant Gene Ontology (GO) classes of up regulated genes in lenalidomide include: antigen processing and presentation (11 genes; $P = 1.9E-16$), immune
25 response (37 genes; $P = 3.9E-13$) and cell death (55 genes; $P = 1.0E-10$) (Tables 4 and 5). Lenalidomide and pomalidomide effects on cell cycle and gene expression profiles in U266 were abrogated by knockdown of CRBN using 4 different siRNAs (Figures 5A – 5C).

6.9 CRBN levels are decreased in multiple myeloma cells resistant to lenalidomide or pomalidomide

Although the development of lenalidomide-based therapies has improved significantly clinical responses, most multiple myeloma patients eventually relapse or become refractory to their therapeutic regimens. Multiple myeloma cell lines resistant to the anti-proliferative effects of lenalidomide have been developed in order to evaluate the potential mechanisms responsible for lenalidomide resistance.

Due to the relevance of CRBN for the anti-proliferative response of lenalidomide, pomalidomide and other immunomodulatory the levels of CRBN protein was compared in matched pairs of parental sensitive lines with acquired lenalidomide- or pomalidomide-resistance cells. Consistent with CRBN being required for the anti-proliferative activity of lenalidomide and pomalidomide, the levels of CRBN protein were significantly lower in the pomalidomide-resistant cells line DF15R and the lenalidomide-resistant cells, H929 R10-1, H929 R10-2, H929 R10-3, H929 R10-4 and MM1/R compared to the matched parental lines (Table 2).

Table 2. Fold difference of CRBN protein level between lenalidomide- or pomalidomide-resistant cells and the matched parental cells

	CRBN protein fold decrease relative to matched parental lines
DF15R	7.16E-07
H929 R10-1	0.503989
H929 R10-2	0.295482
H929 R10-3	0.459599
H929 R10-4	0.659341
MM1/R	0.172249

6.10 Genomic DNA sequencing of CRBN, DDB1 and GSK3 from human cell lines and primary cells

The target genes CRBN, DDB1, and GSK3B were sequenced from a variety of human cell lines and primary cells. Sequencing of the CRBN gene identified mutations in lenalidomide resistant human cell lines which are absent in parental cells.

Sequence enrichment, NexGen sequencing, and data analysis yielded the results shown in Tables 3-6 below. Each table lists the cell lines from which the data are derived. The reference

nucleotide refers to the wild type nucleotide at that position. “Coverage” refers to the total number of reads at that position. The overall mutation score (“Score”) is based on the concept of Phred scores with a maximum value of 30, meaning the probability that the mutations call is wrong is 1/1000; 20, 1/100; and 10, 1/10. A Score, however, does not necessarily mean that the mutation is more than likely a false mutation. A low Score implies only that the mutation cannot be called a true mutation with absolute certainty. “Mutation Call” denotes the nucleotide change. For example, T > TG indicates a heterozygous change from reference T to T and G. T > G indicates a homozygous change from reference T to G. The term “c.” refers to the coding sequence while “IVS” refers to an intron variant. The “Amino Acid Change” column uses similar notation to the “Mutation Call” column.

Results

In the large-scale sequence analysis of 27 cell lines, more intronic mutations were found than exonic mutations. The multiple myeloma cell line, ANBL-6, showed a mutation in CRBN of the lenalidomide-resistant line that was not seen in the wild type. ANBL-6.wt was treated only with DMSO and the CRBN coding sequence exactly matched the reference. However, in the ANBL-6.R10R which has resistance to 10 μ M lenalidomide, a SNP in the coding region c.745C>CA caused an amino acid change 249D>YD in the protein. This amino acid change mapped to the DDB1 binding domain of CRBN.

A silent mutation found in the CRBN coding region of HepG2 and JeKo-1 was heterozygous c.735A>AG with KMS-12-BM as homozygous c.735A>G. The cell line, OPM2, had a different silent mutation in the coding region of CRBN, c.1209C>CT. No amino acid changes resulted from these SNPs.

The sequencing results for DDB1 uncovered a SNP in the coding region c.909T>TA which resulted in an amino acid change 303E>ED in both ANBL-6.wt and ANBL-6.R10R cell lines. A different SNP was found in the Jurkat cell line, with a mutation c.2143C>CT in the DDB1 coding region that changed the amino acid sequence 715V>VI.

A silent mutation found in the DDB1 coding region of ANBL-6.wt, ANBL-6.R10R, and SH-SY5Y at c.153G>GA did not change the amino acid sequence. A sample of PBMC from a healthy donor also had a silent mutation in the DDB1 coding region c.2265G>GA that did not change the amino acid sequence.

One GSK3 β mutation was found in the PMBC sample. The mutation in the coding region c.1187C>CT resulted in an amino acid change 396R>RQ. This mutation did not map to the kinase domain. Other mutations are shown in Table 11. Most of these have low coverage. This could be due to primers targeting these regions and/or sequencing error.

5 While no consistent mutations in CRBN, DDB1 and GSK3- β were found to correlate with resistance to lenalidomide or pomalidomide in these MM cell lines, sporadic mutations such as the 249D>YD CRBN mutation observed in lenalidomide-resistant ANBL-6 MM cells exemplify the type of polymorphism which might occur in such genes within the clinical setting, and which might constitute a mechanism of resistance. Further studies would be needed to
10 confirm or refute this hypothesis.

Table 3 CRBN Mutations

Cell line	Mutation Call	Amino Acid Change
OPM2	c.1209C>CT	403T>TT ^a
HepG2	c.735A>AG	245Y>YY ^a
JeKo-1	<u>c.735A>AG</u>	245Y>YY ^a
KMS-12-BM	c.735A>G	245Y>Y ^a
ANBL-6.R10R	<u>c.745C>AC</u>	249D>YD ^b
KMS-12-BM	IVS1148+21_1148+22insA	
SH-SY5Y	IVS1148+21_1148+22insA	
SKMM2	IVS1148+21_1148+22insA	
Jurkat	IVS1148+23delA	
EJM	IVS1329+18_1329+19insAACT	
H929_R10-4	<u>IVS1329+18_1329+19insAACT</u>	
JJN-3	IVS1329+18_1329+19insAACT	
OPM2	IVS1329+18_1329+19insAACT	
SH-SY5Y	IVS1329+18_1329+19insAACT	
MM.1S.R10R	IVS175-9A>AG	
MM.1S.wt	<u>IVS175-9A>AG</u>	
PBMC	<u>IVS175-9A>AG</u>	
HepG2	IVS175-9A>AG, IVS1148+21_1148+22insA	
ANBL-6.wt	<u>IVS175-9A>G</u>	
EJM	IVS175-9A>G	
H929_D1	<u>IVS175-9A>G</u>	
H929_R10-1	<u>IVS175-9A>G</u>	
H929_R10-2	<u>IVS175-9A>G</u>	

H929_R10-3	<u>IVS175-9A>G</u>	
H929_R10-4	<u>IVS175-9A>G</u>	
H929-1uM-CC5013	IVS175-9A>G	
JeKo-1	<u>IVS175-9A>G</u>	
JJN-3	IVS175-9A>G	
Jurkat	IVS175-9A>G	
KMS-12-BM	IVS175-9A>G	
OPM2	IVS175-9A>G	
RPMI-8226	IVS175-9A>G	
SH-SY5Y	IVS175-9A>G	
SK-Hep1	IVS175-9A>G	
SKMM2	IVS175-9A>G	
U266_B1	<u>IVS175-9A>G</u>	
ANBL-6.R10R	<u>IVS175-9A>G, IVS1148+21 1148+22insA</u>	
U87	<u>IVS175-9A>G, IVS1148+21 1148+22insA, IVS528-29 528-28insCT</u>	
H929	<u>IVS175-9A>G, IVS1329+18 1329+19insAACT</u>	
H929-0.1uM-CC4047	IVS175-9A>G, IVS1329+18_1329+19insAACT	
H929-DMSO	IVS175-9A>G, IVS528-29_528-28insCT	
KMS-12-BM	IVS528-29_528-28insCT	
SKMM2	IVS528-29_528-28insCT	
U266_B1	<u>IVS528-29 528-28insCT</u>	

a = silent mutation; b = region required for DDB1 interaction

Table 4 DDB1 Mutations

Cell line	Mutation Call	Amino Acid Change
ANBL-6.R10R	<u>c.153G>AG</u>	51P>PP ^a
ANBL-6.wt	<u>c.153G>AG</u>	51P>PP ^a
SH-SY5Y	c.153G>AG	51P>PP ^a
Jurkat	c.2143C>CT	715V>VI
PBMC	<u>c.2265G>AG</u>	755S>SS ^a
ANBL-6.R10R	<u>c.909T>AT</u>	303E>DE
ANBL-6.wt	<u>c.909T>AT</u>	303E>DE
ANBL-6.wt	<u>IVS1123-29A>G</u>	
EJM	IVS1123-29A>G	
H929	<u>IVS1123-29A>G</u>	
H929_R10-3	<u>IVS1123-29A>G</u>	
H929-1uM-CC5013	IVS1123-29A>G	

HepG2	IVS1123-29A>G	
JeKo-1	<u>IVS1123-29A>G</u>	
MM.1S.wt	<u>IVS1123-29A>G</u>	
OPM2	IVS1123-29A>G	
SK-Hep1	IVS1123-29A>G	
U266_B1	<u>IVS1123-29A>G</u>	
ANBL-6.wt	<u>IVS1123-30C>T</u>	
EJM	IVS1123-30C>T	
H929	<u>IVS1123-30C>T</u>	
H929_R10-3	<u>IVS1123-30C>T</u>	
H929-1uM-CC5013	IVS1123-30C>T	
HepG2	IVS1123-30C>T	
JeKo-1	<u>IVS1123-30C>T</u>	
MM.1S.wt	<u>IVS1123-30C>T</u>	
OPM2	IVS1123-30C>T	
SK-Hep1	IVS1123-30C>T	
U266_B1	<u>IVS1123-30C>T</u>	
PBMC	<u>IVS1225+30T>C</u>	
MM.1S.R10R	IVS1225+30T>CT	
MM.1S.wt	<u>IVS1225+30T>CT</u>	
RPMI-8226	IVS1225+30T>CT	
JJN-3	IVS1862-26A>AT	
OPM2	IVS1862-26A>AT	
H929-0.1uM-CC4047	IVS1862-27A>AC	
JJN-3	IVS1862-27A>AC	
OPM2	IVS1862-27A>AC	
SH-SY5Y	IVS1862-27A>AC	
H929	<u>IVS2278-26T>GT</u>	
H929-1uM-CC5013	IVS2278-26T>GT	
Jurkat	IVS2278-26T>GT	
KMS-12-BM	IVS2278-26T>GT	
OPM2	IVS2278-26T>GT	
ANBL-6.wt	<u>IVS2278-27C>CT</u>	
EJM	IVS2278-27C>CT	
H929	<u>IVS2278-27C>CT</u>	
H929_R10-2	<u>IVS2278-27C>CT</u>	
H929-1uM-CC5013	IVS2278-27C>CT	
JJN-3	IVS2278-27C>CT	

Jurkat	IVS2278-27C>CT	
KMS-12-BM	IVS2278-27C>CT	
OPM2	IVS2278-27C>CT	
PBMC	<u>IVS2278-27C>CT</u>	
SH-SY5Y	IVS2278-27C>CT	
SK-Hep1	IVS2278-27C>CT	
SKMM2	IVS2278-27C>CT	
U266_B1	<u>IVS2278-27C>CT</u>	
U87	<u>IVS2278-27C>CT</u>	
ANBL-6.wt	<u>IVS2278-28C>T</u>	
H929	<u>IVS2278-28C>T</u>	
H929_R10-2	<u>IVS2278-28C>T</u>	
H929_R10-3	<u>IVS2278-28C>T</u>	
H929-1uM-CC5013	IVS2278-28C>T	
Jurkat	IVS2278-28C>T	
KMS-12-BM	IVS2278-28C>T	
OPM2	IVS2278-28C>T	
PBMC	<u>IVS2278-28C>T</u>	
SH-SY5Y	IVS2278-28C>T	
SKMM2	IVS2278-28C>T	
U266_B1	<u>IVS2278-28C>T</u>	
U87	<u>IVS2278-28C>T</u>	
JJN-3	IVS2661+6C>CT	
PBMC	<u>IVS2832+6C>CT</u>	
RPMI-8226	IVS2832+6C>CT	

a = silent mutation

Table 5 GSK3β Mutations

Cell line	Mutation Call	Amino Acid Change
PBMC	<u>c.1187C>CT</u>	396R>RQ
Jurkat	IVS282+3delT	
U266_B1	<u>IVS366+29A>AG</u>	
H929	<u>IVS366+29A>G</u>	
Jurkat	IVS909+11delA	

Table 6 Other Mutations

Chr Y: LOC100288025
Chr M: ATP6, COX1, CYTB, ND1, ND4, ND4L, ND5
Chr 2: C2orf67

Chr 1: ITLN1
Chr 19: KLK10
Chr 17: KRT15
Chr 14: PRMT5, FMNL1
Chr 9: NOTCH1

6.11 Study of the relationship between drugs and the ubiquitin proteasome system

Specific antibodies for poly-ubiquitin chains were used to study the effect of immunomodulatory drugs on global levels of ubiquitination. H929 cells were treated with lenalidomide (1 μ M), pomalidomide (1 μ M) or proteasome inhibitor MG132. After 30 minutes cells were process for immunofluorescence. Global levels of K63-linked polyubiquitination were quantified by Cellomics. As shown in Figures 7A & 7B, immunomodulatory drugs decrease total K48-linked polyubiquitination but not K-63-linked ubiquitination in H929.

6.12 Study of the effect of drugs on global changes of ubiquination and protein abundance

The effects of lenalidomide and pomalidomide on protein ubiquination were investigated using CST Ubiscan technology. Treated samples were sent to Cell Signaling Technology for ubiquitinated peptide enrichment and quantification by LC/MS/MS. Raw intensity was used for this analysis to identify peptides that are significantly regulated by the drugs, or the drugs with proteasome inhibitor MG132. Analysis showed that compared with MG132, the effects of lenalidomide and pomalidomide on ubiquitinated peptides are small. In the MG132 combo, more ubiquitinated peptides are observed (compared with their controls). 162 unique ubiquitinated peptides were significantly up-regulated by lenalidomide and pomalidomide alone, or with MG132 at 1 hour or 4 hours. These peptides correspond to 176 unique proteins. Top few groups are: nucleosome, chromatin, protein-DNA complex assembly, Histone H2A. Among the 176 proteins, we found five proteins that belong to "ubiquitin-protein ligase activity" category, they are MDM2, HERC2, UBE2D3 (only by pomalidomide), UBE2N (lenalidomide only), UBE2M (both). Results for hits categorized by conditions are shown in Figures 8 & 9 (without MG132) and Figures 10 & 11 (with MG132).

6.13 CRBN knockdown effect on drug induced TNF α and IL-2 in primary T-cells

In these studies, human T cells were isolated from blood and treated with 1 µg/ml PHA-L at 37°C. After 24 hr stimulation T cells were subjected to siRNA transfection with the indicated siRNAs. Knockdown efficiencies were analyzed by qRT-PCR after 24 h transfections and the remaining transfected cells seeded in 96-well plate s pre-bound with OKT3 and treated with DMSO or 1 and 10 µM thalidomide, lenalidomide, pomalidomide and phthalimide in duplicate at 37°C for 48 hours. After 48 hours, the supernatants were collected and tested for TNFα and IL-2 production by ELISA (Figures 12A – 12D). This data indicates that siRNA knockdown of CRBN abrogates drug-induced TNFα and IL-2 production in anti-CD3-stimulated primary human T cells.

6.14 Knocking down Cul4A and Cul4B together partially abrogates lenalidomide, pomalidomide, or Compound B-induced TNFα and IL-2 induction in T cells

Cul4A knockdown efficiency prior to drug treatment was measured. Cul4A gene expression was knocked down by 82% and 76% by Cul4A siRNA-1 and Cul4A + Cul4B siRNA, respectively (Figure 13A). Cul4B gene expression was suppressed by 70% and 63% by Cul4B and Cul4A + Cul4B siRNA, respectively. The individual knock down of Cul4A or Cul4B had no effect on T cell TNF-α or IL-2 production induced by 10 µM lenalidomide, pomalidomide or Compound B (Figures 13 B & C). However, the double knockdown of Cul4A and Cul4B together resulted in a significant but partial reversal of the compound-induced elevation of TNF- production due to lenalidomide, pomalidomide, and Compound B (Figure 13B). There was a trend of a reversal of IL-2 induction when Cul4A and Cul4B were knocked down (Figure 13C). These data suggest that lenalidomide, pomalidomide, and Compound B-mediated T cell costimulation is dependent on the expression of Cul4A and Cul4B, and that these proteins serve redundant functions in the T cell.

6.15 CRBN expression and sensitivity to lenalidomide in lymphoma cells

The antiproliferative activity of lenalidomide versus baseline CRBN expression was studied in diffuse large B-cell lymphoma (DLBCL) cell lines. The following DLBCL cell lines were evaluated for sensitivity to lenalidomide: OCI-Ly10-NCI, U2932, OCI-Ly-3, DB, RIVA, TMD8, Toledo, OCI-Ly-19, Pfeiffer, WSU-DLCL2, Karpas-1106P and SU-DHL-4. Results are shown in Figure 14.

6.16 Preparation of CRBN-DDB1 complex

Primers were designed for cloning of CRBN into pBV-ZZ-HT-LIC and pBV-notag-LIC. Two primers were prepared, “CRBN_For” and CRBN_Rev.”

CRBN_For: GTGCCGCGTGGCTCCATGATGGCCGGCGAAGGAGATCA

5 CRBN_Rev: GCTTCCTTTTCGGGCTTATTACAAGCAAAGTATTACTT

The primers were used to amplify the CRBN gene from a cDNA library. The product was gel purified and treated with T4 DNA polymerase in the presence of TTP only to make single-stranded ends compatible for ligation independent cloning. The CRBN DNA was then annealed to pBV-ZZ-HT-LIC to create CRBN_034 (Figure 15A).

10 For DDB1, two primers were prepared, “DDB1_For” and “DDB1_Rev.”

DDB1_For: TCGGGCGCGGCTCTCGGTCCGAAAAGGATGTCGTACAACCTACGTGGTAAC
(SEQ ID NO:2)

DDB1_Rev:

GCTTCCTTTTCGGGCTTATTTTTCGAACTGCGGGTGGCTCCAATGGATCCGAGTTAGCTCCT
15 (SEQ ID NO:3)

The DDB1_Rev adds a StrepTag at the C-termini of DDB1. The DDB1 gene was amplified from a cDNA library, gel purified, and treated with T4 polymerase in the presence of TTP. The DDB1 gene was then annealed to pBV-notag-LIC to create the plasmid DDB1_004 (Figure 15B).

20 *Expression of constructs in baculovirus and purification*

The constructs could were tested for expression in insect cells. Recombinants pBV-HT-LIC and pBV-GST-LIC plasmids were transformed into DH10Bac to produced bacmids. Integrity of the recombination was followed by a blue-white screen and PCR. The recombinant bacmids were used to transfect 9 x 10⁵ Sf9 adherent cells per well in serum free-Grace media.

25 After the transfection, fresh Grace media containing antibiotics and glutamine was added.

Infection was followed by observation of the cell monolayers under microscope. After 5 to 7 days, the supernatants were saved (P1 virus) and the pellets were analyzed for recombinant protein expression. Virus amplification was in 24 deep well plates with 4 ml of 2 x 10⁶ Sf9 cells/ml per well. Aliquots were removed to assess the kinetics of protein expression. After 4
30 days, the plate was centrifuged, the supernatant were saved (P2 virus) and the pellets were analyzed by mini scale purification of the tagged proteins. The viruses were finally amplified in

750 ml of Grace media containing antibiotics and glutamine and the supernatants saved as P3 virus. Pellets were saved and purified using either the AKATxpress or the AKTA purifier from Amersham Biosciences.

Purification

5 Cell pastes containing CRBN-DDB1 were lysed in a buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 20 mM Imidazole, 10% Glycerol, and 2 mM DTT and protease inhibitors. The lysate was then cleared by centrifugation. The tagged proteins were then purified from the supernatants using the AKTA Express from Amersham Biosciences. Five ml HisTrap HP columns were used for the affinity step while a 16 mm X 60 cm Sephacryl S-200 HR was used
10 for the size exclusion steps. After loading the column, the column was washed with 20 volumes of lysis buffer, then 10 column volumes of 50 mM Tris pH 8.0, 1000 mM NaCl, 40 mM Imidazole, 10% Glycerol, and 2 mM DTT. Elution of the bound proteins was performed with lysis buffer containing 500 mM Imidazole. Eluted proteins were directly injected into the gel filtration column equilibrated in 25 mM Tris pH 8.0, 200 mM NaCl, 5 % Glycerol, and 2 mM
15 DTT. Fractions were analyzed by 4-20 % SDS polyacrylamide gel electrophoresis.

Fractions containing both CRBN and DDB1 were pooled and digested with Thrombin to remove the ZZ-HT tag from CRBN. Digestion was carried at 4°C for 5-6 hours with 1:2000 (weight/weight) of thrombin and CRBN-DDB1. Cleaved CRBN-DDB1 was diluted in 25 mM Tris pH 8.0, 5 % Glycerol, and 2 mM DTT, and loaded onto an 8 ml MonoQ column
20 (Amersham-Pharmacia) equilibrated in 25 mM Tris pH 8.0, 75 mM NaCl, 5% Glycerol, and 2 mM DTT. After loading the column was washed with 2 volumes of 25 mM Tris pH 8.0, 75 mM NaCl, 5 % Glycerol and 2 mM DTT, and the bound proteins were eluted with a gradient from 75 mM to 400 mM in 25 mM Tris pH 8.0, 5 % Glycerol and 2 mM DTT

A final gel filtration was done to polish the CRBN-DDB1 complex. The fraction pooled
25 from the MonoQ were loaded onto a 140 ml S200HR Gel filtration column and ran in 25 mM Tris pH 8.0, 200 mM NaCl, 5 % Glycerol, and 2 mM DTT. Fractions were analyzed and positive fractions were pooled and concentrated to approximately 15 mg/ml. Aliquots were stored at -80°C.

6.17 Ubiscan ubiquitination experiments

30 The results of 1 hour and 4 hour ubiquitination experiments are shown in Figures 17-22, which demonstrate that certain peptides are regulated by lenalidomide and/or pomalidomide.

The tables of Figure 23 show Ubiscan data results for lenalidomide, pomalidomide and Compound B. Ub-proteins IKZF3, RPL19, PCM1 and NEDD8 were commonly increased in abundance by Rev and Pom in U266 and Compound B in T cells. Proteins GNB2L1 and HNRNPR were commonly decreased in abundance by Rev and Pom in U266 and Compound B in T cells.

Treatment of T cells with Compound B resulted in a greater abundance of two ubiquitinated peptides (vs. DMSO control), SECTM1 and ZC3H15.

Proteins in common with lenalidomide, pomalidomide and Compound B: IKZF3, RPL19, PCM1, NEDD8, GNB2L1, and HNRNPR.

Table 7 Final Ubiscan hits for lenalidomide and pomalidomide (1hr)

ID	Gene Name	Peptide Sequence
1	147 G3BP2	QYYTLLNK*APEYLHR
2	904 COPS6	QVCEIIESPLFLK*LNPM#TK
3	905 COPS6	QVCEIIESPLFLKLNPM#TK*
5	1137 VCIPI1	VGDVQGQESQLPTKIILTGQK*
9	1582 HYOU1	FFGDSAASM#AIK*NPK
10	2541 MCM7	FLQEFYQDDELGK*K
13	3043 CCT3	SM#M#K*MLLDPMGGIVM#TNDGNAILR
14	3226 LMNB2	LSSDQNDK*AASAAR 278
16	3910 C12orf51	LSPYLEDVSGGMWPVVHIQK*KNTK
18	3992 EDEM3	ATGDPYYLEVVGK*TLIENLNK
25	4982 RAB28	VVK*ADIVNYNQEPM#SR
33	7093 HNRNPUL1	IGWSLDSCSTQLGEEPFSYGYGGTGK*K
35	7556 ABCF2	YGLTGK*QQVSPIR
37	7740 DNAJC1	ALPHLIQDAGQFYAKYK*
38	7798 GNAS	VLTSGIFETKFQVDK*VNFHM#FDVGGQRD ER
39	8193 SLC16A1	ASLEK*AGK
42	10418 RPL19	HMGIGK*R
43	10556 RPL36	AMELLKVSK*
44	10565 RPL4	MFAPTK*TWR
46	11831 UBE2Q1	ELK*LLESIFHR
47	12009 ARMC6	NLVAHGQAFSK*PILDLGAEALIM#QAR
50	12698 KLHL7	ISVNSNNVQSLLDAANQYQIEPVK*K
51	12891 NUP37	FCTSAADMK*IR
52	12942 POC5	VVTSAQQK*AGR
54	13152 SNRPE	IM#LK*GDNITLLQSVSN

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Table 8 Final Ubiscan hits for lenalidomide and pomalidomide (MG 1hr)

ID	Gene	Name	Peptide Sequence
1	105	DOK5	K*ASSKGPK
2	324	SHCBP1	AYQDYILADCK*ASEVQEFTAEFLEK
4	389	YWHAE	MK*GDYHR
5	512	FERMT3	ETTLSYYK*SQDEAPGDPIQQLNLK
6	904	COPS6	QVCEIIESPLFLK*LNPM#TK
7	905	COPS6	QVCEIIESPLFLKLNPM#TK*
8	1010	PCM1	LMAAK*QK
9	1112	SMC4	SVAVNPK*EIASK
10	1114	TOPORS	K*IQEQDIINFR
11	1323	HSPA1A	SAVEDEGLK*GK
12	1323	HSPA1B	SAVEDEGLK*GK
13	1452	HSPA8	DISENK*R
14	1623	ATRX	DNRGGIKSK*
15	1679	DUT	IFYPEIEEVQALDDTERGSGGFGSTGK*N
16	1773	H2AFJ	K*GNYAER
17	1773	HIST1H2 AA	17
18	1773	HIST1H2 AC	K*GNYAER
19	1773	HIST1H2 AI	K*GNYAER
20	1773	HIST1H2 AK	K*GNYAER
21	1773	HIST2H2 AA3	K*GNYAER
22	1773	HIST2H2 AB	K*GNYAER
23	1773	HIST2H2 AC	K*GNYAER
24	1863	H2AFX	K*TSATVGPK
25	3146	LMNA	TLEGELHDLRGQVAK*LEAALGEAK
26	3192	LMNA	K*LESTESR
27	3377	TUBA1A	DVNAAIATIK*TK
28	3377	TUBA1B	DVNAAIATIK*TK
29	3377	TUBA1C	DVNAAIATIK*TK
30	3377	TUBA3C	DVNAAIATIK*TK
31	3377	TUBA3D	DVNAAIATIK*TK
32	3377	TUBA3E	DVNAAIATIK*TK
33	3963	DHFR	LTEQPELANK*VDM#VWIVGGSSVYK
34	3984	DHX15	EVDDLGPVGDIK*IIPLYSTLPPQQQQR
37	4111	GBA	LLPHWAK*VVLTDPEAAK
41	4199	IDH3G	NTGK*SIANK

43	4471	PPAT	CGLPYVEVLCK*NR
44	4938	GNB3	GQQK*TV
45	4938	H2AFZ	GQQK*TV
46	5121	VAV1	VLK*YHLLLQELVK
47	5121	VAV3	VLK*YHLLLQELVK
48	5126	VAV1	IDGELK*ITSVER
49	5252	UCK2	VLTSEQKAK*
50	5377	ALDOA	ADDGRFPQVIK*SK
54	5582	SDHA	AFGGQSLKFGK*
59	6046	PSMA7	AITVFSPDGHLFQVEYAQEAVK*K
60	6046	PSMA8	AITVFSPDGHLFQVEYAQEAVK*K
61	6049	PSMA7	AITVFSPDGHLFQVEYAQEAVKK*
62	6049	PSMA8	AITVFSPDGHLFQVEYAQEAVKK*
64	6567	IRAK2	CPIPAFPDSVK*PEKPLAASVR
65	6662	PRKDC	NILEESLCELVAKQLK*
66	6662	LOC73175 1	NILEESLCELVAKQLK*
67	6741	PRKDC	GHDEREHPFLVK*GGEDLR
68	6741	LOC73175 1	GHDEREHPFLVK*GGEDLR
70	7093	HNRNPU L1	IGWSLDSCSTQLGEEPFSYGYGGTGK*K
72	7306	PCBP2	LVVPASQCGSLIGK*GGCK
75	7542	ABCE1	VAETANEEEVKK*
78	7783	GNAL	VLAKG*SK
79	7783	GNAS	VLAKG*SK
80	7939	KCNAB2	IGVGAM#TWSPLACGIVSGK*YDSGIPPYSR
81	8156	PTCH1	DKPIDISQLTK*QR
82	8173	SEMA4A	VCK*NDVGGEK
84	8478	ASCC3	FQALQDNCK*K
85	8779	HMGB2	IK*SEHPGLSIGDTAK
86	8808	IFI16	KK*EVDATSPAPSTSSTVK
88	8973	ILF2	K*ILGQEGDASYLASEISTWDGVIVTPSEK
89	9023	IRF4	QWLIDQIDSGK*YPGLVWENEEK
91	9082	NACA	NILFVITKPDVYK*SPASDTYIVFGEAK
92	9465	SUPT5H	SSVGETVYGGSDLSDDITQQQLPGVK*DPNLWTVK
93	9525	TRIP4	GK*DVEFPNDYPSGCLLGCVDLIDCLSQK
94	9764	EEF1A1	IGYNPDTVAFVPISGWNGDNM#LEPSANM#PWFK*GW K
95	9764	eEF1AL3	IGYNPDTVAFVPISGWNGDNM#LEPSANM#PWFK*GW K
96	9766	EEF1A1	IGYNPDTVAFVPISGWNGDNM#LEPSANM#PWFKGW K*
97	9766	eEF1AL3	IGYNPDTVAFVPISGWNGDNM#LEPSANM#PWFKGW

			K*
98	9794	EEF1A1	AAGAGK*VTK
99	9794	eEF1A1L3	AAGAGK*VTK
106	10407	RPL19	TLSK*EEETKK
107	10409	RPL19	TLSKEEETK*K
108	10411	RPL19	TLSKEEETKK*
111	11082	RPS25	LVSK*HR
112	11397	COPS3	LKAM#DQEITVNPQFVQK*SM#GSQEDDSGNKPSSYS
113	11426	CUL9	ILK*AHGEK
115	11794	UBAP2L	IDLAVLLGK*TPSTMENDSSNLDPSQAPSLAQPLVFSN SK
116	11931	ADRM1	GTTVTPDK*R
120	12423	FAM10A4	AK*SEENTKEEKPDSEK
121	12423	FAM10A5	AK*SEENTKEEKPDSEK
122	12423	ST13	AK*SEENTKEEKPDSEK
123	12447	FAM129A	VLK*QYDYDSSTIR
125	12609	IGJ	FVYHLSDLCK*K
126	12627	IKZF3	SHTVEKPYK*CEFCGR
131	13060	S100A6	EGDKHTLSK*K
132	13212	SSR2	KYDTPK*TK
133	13250	SSX1	SK*AFDDIATYFSK
134	13306	TIPRL	LK*VVPTTDHIDTEKLEK
136	13598	AMBRA1	REPFVVK*TASEM#ER
137	13676	COPG	ALQYTLPESEKPFDLK*SVPLATAPM#AEQR
138	13280	TBCC	GK*DAASSTKVDAAPGIPPAVESIQDSPLPK
139	13281	TBCC	GKDAASSTK*VDAAPGIPPAVESIQDSPLPK

Table 9 Final Ubiscan hits for lenalidomide and pomalidomide (4hr)

ID	Gene	Name	Peptide Sequence
1	236	GNB2L1	LK*TNHIGHTGYLNTVTVSPDGSLCASGGK
2	487	CTNNB1	LLHPPSHWPLIK*ATVGLIR
3	512	FERMT3	ETTLSSYYK*SQDEAPGDPIQQLNLK
5	663	PKP2	TYDMLK*AGTTATYEGR
6	732	HSP90B1	NLGTIAK*SGTSEFLNK
9	1112	SMC4	SVAVNPK*EIASK
10	1863	H2AFX	K*TSATVGPK
11	2508	HIST2H2AB	KTESHKPGK*NK
12	2512	HIST2H2AB	KTESHKPGKNK*
13	2579	NAP1L2	GLIGYVLDTDFVESLPVKVK*
15	3712	TPD52	SFEEK*VENLK
16	3742	VAPA	RYCVRPNSGIIDPGSTVTVSVM#LQPFDYDPNEKSK*
17	3799	AKR1B1	LLLNNGAK*M#PILGLGTWK
18	3916	CHPF	SALTAHPVRDPVHMYQLHK*AFAR
19	3955	DDX24	ATNEGLSLM#LIGPEDVINFKK*

20	4035	FASN	DGLLENQTPEFFQDVCKPK*
23	4181	HMOX1	K*AALEQDLAFWYGPR
25	4347	NANS	QLLPCEMACNEK*LGK
27	4938	GNB3	GQQK*TV
28	4938	H2AFZ	GQQK*TV
29	4968	NET1	IGEATK*PDGTVEQIGHILVSWLPR
31	5672	ANP32E	KLELSDNIISGGLEVLAEK*CPNLTYLNLSGNK
32	5676	CDC25C	SLNQYPALYYPELYILK*GGYR
33	5732	PPP1CB	AK*YQYGGLNSGRPVTTPR
35	6543	CHEK1	M#CGTLPYVAPELLK*R
36	6546	CSNK1A1	LFLIDFGLAK*K
37	6546	CSNK1A1L	LFLIDFGLAK*K
38	6563	IRAK1	GTLAYLP EEYIK*TGR
40	6843	PRKAG2	KK*DVSSPGSGGK*K*NASQK*
41	6858	CPSF3L	VNCYM#PANGETVTLPTSPSIPVGISLGLLK*R
42	7344	RARS	SDGGYTYDTSDLAAIK*QR
43	7672	C7orf42	QSNPEFCPEK*VALAEA
44	7717	CPNE1	SEVIK*NNLNPTWK
46	8137	NUP88	IYSLREPQTPTNVII LSEAEEESLVLNK*GR
47	8331	TCIRG1	QEENK*AGLLDLPDASVNGWSSDEEK
48	8723	FAF1	K*SPM#M#PENAE NEGDALLQFTA EFSSR
49	8734	FUBP3	ITGDAFK*VQQAR
54	9253	PSMC3	LAGPQLVQM#FIGDGAK*LVR
55	9526	TSG101	ASLISAVSDK*LR
56	9794	EEF1A1	AAGAGK*VTK
57	9794	eEF1AL3	AAGAGK*VTK
58	9933	EIF2S1	ADIEVACYGYEGIDAVK*EALR
62	10690	RPL9	K*FLDGIYVSEK
64	11010	RPS2	AEDK*EWMPVTK
65	11010	LOC645018	AEDK*EWMPVTK
66	11585	NAE1	CINITK*QTPSFWILAR
67	11831	UBE2Q1	ELK*LLESIFHR
68	12110	C13orf40	KPDLRIIEQEEK*
70	12242	COMMD4	LEVAAAPGTPAQPVAM#SLSADK*FQVLLAELK
71	12320	DDIT4	GALLDVCVEQGK*SCHSVGQLALDPSLVPTF
72	12458	FAM60A	TPVFSFLDLTYWK*R
73	12545	HLA-E	GYEQFAYDGK*DYLT LNEDLR
74	12758	MAGEA3	AREPVTK*AEM#LGSVVGNWQYFFPVIFSK
75	12758	MAGEA6	AREPVTK*AEM#LGSVVGNWQYFFPVIFSK
76	12865	NHP2	IK*ADPDGPEAQAEACSGER
78	13290	TBL3	LWTIK*NNECVR
79	13930	NEDD8	QMNDEK*TAADYK
80	403	YWHAZ	YLAEVAAGDDK*K
81	805	SHISA5	SQPPYNPAYM#DAPK*AAL
82	2089	HIST1H1A	GTLVQTK*GTGASGSFK
83	2089	HIST1H1B	GTLVQTK*GTGASGSFK

84	2089	HIST1H1C	GTLVQTK*GTGASGSFK
85	2089	HIST1H1D	GTLVQTK*GTGASGSFK
86	2089	HIST1H1E	GTLVQTK*GTGASGSFK
87	13902	NEDD8	LIYSGK*QMNDEK
88	14057	RPS27A	K*IQDK*EGIPPDQQR
89	14057	RPS27AP5	K*IQDK*EGIPPDQQR
90	14057	UBA52	K*IQDK*EGIPPDQQR
91	14057	UBB	K*IQDK*EGIPPDQQR
92	14057	UBC	K*IQDK*EGIPPDQQR

Table 10 Final Ubiscan hits for lenalidomide and pomalidomide (MG 4hr)

ID	Gene Name		Peptide Site
1	11	PLAA	FIIDNTK*GQM#LGLGNPSFSDPFTGGGR
2	259	HGS	ACGQIFCGK*CSSK
3	260	HGS	ACGQIFCGKCSSK*
4	358	UBE2M	VGQGYPHDPPK*VK
5	361	UBE2M	VGQGYPHDPPKVK*
6	533	IL32	GDK*EELTPQK
7	826	ANXA7	AM#K*GFGTDEQAIVDVVANR
8	917	CP110	NKMLGTSSKESEELLK*SK*
9	1112	SMC4	SVAVNPK*EIASK
10	1146	ANP32B	IFGGLDM#LAEKLPNLTHLNLSGNKCLK*
11	2579	NAP1L2	GLIGYVLDTDFVESLPVKVK*
12	2591	PURA	FFFDVGSNK*YGVFM#R
13	2768	VCP	KAFEEAEK*NAPAIIFIDELDAIAPKR
14	2791	VCP	ELQELVQYPVEHPDKFLK*
15	2929	ACTB	DIK*EKLCYVALDFEQEMATAASSSSLEK
16	2929	ACTG1	DIK*EKLCYVALDFEQEMATAASSSSLEK
17	2929	POTEE	DIK*EKLCYVALDFEQEMATAASSSSLEK
18	3231	LSP1	QEM#LLSLK*PSEAPELDEDEGFGDWSQRPEQR
19	3281	NES	TSLSFQDPK*LELQFPR
20	3575	AUP1	FPSSGPVTPQPTALTFAK*SSWAR
21	3588	MAN1A1	GYAWGLNELK*PISK

22	3589	MAN1A1	K*GSGPAALR
23	3712	TPD52	SFEEK*VENLK
24	3740	VAPA	YCVRPNSGIIDPGSTVTVSVM#LQPFDPNEK*SK
27	4483	PPIA	VSFELFADK*VPK
29	4655	TPI1	ELASQPDVDGFLVGGASLK*PEFVDIINAKQ
30	4656	TPI1	KQSLGELIGTLNAAK*VPADTEVVCAPPTAYIDFAR
31	4938	GNB3	GQQK*TV
32	4938	H2AFZ	GQQK*TV
33	5308	ALDOA	VDK*GVVPLAGTNGETTTQGLDGLSER
34	5459	MTX1	FTGAPLK*VHKISNPWQSPSGTLPALR
35	5573	PTRH2	TQIAPGSQTVLGIGPGPADLIDKVTGHLK*LY
36	5640	MYL6	VFDK*EGNGTVMGAEIR
37	5792	PPP3CA	HLTEYFTFK*QECK
38	5792	PPP3CB	HLTEYFTFK*QECK
39	5810	PPP3CC	SQATGFPSLITIFSAPNYLDVYNNK*AAVLK
40	6427	UCHL5	TLAEHQQLIPLVEKAK*
41	6449	USP15	GPSTPNVK*NSNYCLPSYTAYK
42	6558	HUNK	K*PEPHQPGPGSTGIPHK*EDPLMLDM#VR
45	6861	CPSF6	KTTQSGQMSGEGK*AGPPGGSSR
46	7088	HNRNPR	IK*ALLER
47	7088	SYNCRIP	IK*ALLER
48	7186	MORC3	STNQQTATDVSTSSNIEESVNHM#DGESLK*LR
49	7240	PABPC1	VVCDENGSK*GYGFVHFETQEAAER
50	7522	ABCE1	STALK*ILAGK
51	7672	C7orf42	QSNPEFCPEK*VALAEA
52	8030	MLC1	K*GSMDSANILDEVPPPAR
53	8239	SLC35F2	TAEPAESSVPPVTSIGIDNLGLK*LEENLQETHSAVL
54	8370	TMEM57	KHNLGINNNILQPVDISKIIEIYM#ENHINSK*
56	9215	POLR2L	CFTCGKIVGNK*WEAYLGLLQAEYTEGDALDALGLKR
57	9354	RSF1	AQIDPVLLK*NSSQQDNSSR
58	9398	SP140	MK*ESPGSQCCQESEVLER

59	9468	TBL1XR1	DK*LAQQQAAAAAAAAAAAAASQQGSAK
61	10524	RPL30	KSEIEYYAM#LAK*TGVHHYSGNNIELGTACGK*YYR
62	11010	RPS2	AEDK*EWMPVTK
63	1101	LOC645018	AEDK*EWMPVTK
64	11435	DCUN1D1	QFM#IFTQSSEK*TAVSCLSQNDWK
65	11510	MDM2	ENWLPEDKGKDKGEISEK*
66	11580	MIB1	SSEDATDDISSGNIPVLQK*DKDNTNVNADVQK
67	11581	MIB1	SSEDATDDISSGNIPVLQKDK*DNTNVNADVQK
68	11830	UBE2O	STDSQCGTVIDVNIDCAVK*LIGTNCHYPVNSK
69	11969	ANKRD13A	LTLDLM#KPK*
71	12110	C13orf40	KPDLRIIEQEEK*
72	12127	C19orf43	QKTEDEVLTSK*GDAWAK
73	12129	C19orf43	QKTEDEVLTSKGDAWAK*
74	12171	CAPN8	LAGKDSEITANALK*
75	12259	COPS8	K*PVAGALDVSFNKFIPLSEPAPVPPIPNQQLAR
76	12322	DDIT4	K*LYSSEQLLIEEC
77	12432	FAM114A1	SVLTGGGLDALEFIGK*
78	12564	HSPBP1	AMQQQVQK*LK
79	12929	PLIN2	GAVTGSVEK*TK
80	12930	PLIN2	GAVTGSVEKTK*
81	13264	STRBP	M#VLLPVM#K*FPTYVPVPHYSFF
82	13561	WDR6	MVK*VDPETR
83	13849	VAMP8	NLQSEVEGVK*NIMTQNVER
84	2097	HIST1H1A	K*ALAAAGYDVEKNNSR
85	2097	HIST1H1C	K*ALAAAGYDVEKNNSR
86	2097	HIST1H1D	K*ALAAAGYDVEKNNSR
87	2097	HIST1H1E	K*ALAAAGYDVEKNNSR
88	2097	HIST1H1T	K*ALAAAGYDVEKNNSR
89	3242	MYO18A	INSLQDMVTK*YQKR
90	4677	UAP1	LTLISK*AGQEHLR
91	8262	SLC3A2	IK*VAEDEAEAAAAAK

6.18 Efficacy of lenalidomide in activated B-cell like subtype DLBCL is dependent upon expression of IRF4 and CRBN

Cell proliferation assay

5 Cell proliferation was assessed using the ^3H -thymidine incorporation assay. Briefly, logarithmically growing DLBCL cells were cultured in 96-well culture plates in complete media with the indicated concentration of lenalidomide or DMSO control. Following incubation at 37°C for 5 days, 1 μCi ^3H -thymidine (GE Healthcare Biosciences, Piscataway, NJ) was added to each well for the final 5 hours of incubation. Cells were then harvested onto UniFilter GF/C filter
10 plates (PerkinElmer, Waltham, MA) using a cell harvester (Tomtec, Hamden, CT) and the plates were allowed to dry overnight. The ^3H -thymidine incorporation of each well was then measured using a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard BioScience, Meriden, CT). The percent inhibition of cell proliferation was calculated and normalized to DMSO control.

Protein expression analysis

15 Cells were treated with test compounds or 0.1% DMSO for indicated times. Following incubation, cells were collected, pelleted with centrifugation, and immediately lysed in 0.1 ml lysis buffer containing 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDS, 1 mM DTT, 1 mM Na_3VO_4 , plus Complete protease inhibitor cocktail (Roche Applied
20 Science, Indianapolis, IN), then processed with a Qiashredder™ (Qiagen, Valencia, CA) for 1 minute and frozen on dry ice. Samples were diluted with 6 $\times$ SDS sample buffer and then boiled for 5 min. Approximately 30 μl of this mixture was loaded per lane on a Criterion Precast 4–12% Tris-HCl gel (Bio-Rad, Hercules, CA), electrophoresed, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 1 hour at room
25 temperature using blocking buffer (LI-COR Biosciences, Lincoln, Nebraska), then incubated overnight at 4°C with antibodies against either BCL-10, IRF4, CRBN or β -actin. Membranes were washed and incubated with IRDye Secondary Antibodies (1:30,000) for 1 hour at room temperature. A standard protocol was then followed for signal detection, using the Odyssey® Infrared Imaging System and software (LI-COR Biosciences, Lincoln, NE).

NF-κB activity assays

Logarithmically growing DLBCL cells were treated with test agents as indicated. Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) and protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL).

5 Detection of NF-κB activity was performed using a sensitive oligo-based colorimetric enzyme-linked immunosorbent assay (ELISA) method, according to the instructions of the manufacturer (Active Motif). Briefly, nuclear extracts of DLBCL cells were hybridized to 96-well plates coated with wild-type DNA oligonucleotides containing a single copy of the NF-κB consensus binding sequence. Bound NF-κB protein was then detected with antibodies specific for p50, p65
10 (Rel A) or p70 subunits. A secondary antibody conjugated to horseradish peroxidase was added and plates were then read by spectrophotometry at 450 nm with a reference wavelength of 655 nm. NF-κB activity was calculated based on OD450/655 nm.

For the NF-κB -driven luciferase reporter gene assay, cells were transfected with the pGL4.32 [luc2P/NF-κB-RE/Hygro] plasmid (Promega, Madison, WI) using Nucleofector kit V
15 and program 013 according to the manufacturer's protocol (Amaxa Biosystems, Gaithersburg, MD). After 24 hours of transfection, cells were treated with test agents for 2 days and lysates were prepared using the Dual-Glo® Luciferase Assay System (Promega). Luciferase activities of each sample were measured using a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard BioScience).

Real-time quantitative reverse transcriptase-PCR analysis

After 48 hours of cell treatment, total RNA was purified with RNeasy® Mini Kits using QiaCube™ system (Qiagen Inc., Valencia, CA). Real-time quantitative RT-PCR with 25–100 ng of total RNA was performed using the reverse transcription kit and Taqman® PCR probes specific for the genes of interest according to standard methods (Applied Biosystems Inc.). The
25 quantity of product was normalized to glyceraldehyde-3-phosphate dehydrogenase as the endogenous housekeeping gene. Fold increase of gene expression was calculated using comparative Ct method ($2^{-\Delta\Delta C_t}$).

Electroporation for overexpression and knockdown of IRF4

To knockdown IRF4, cells were transfected with *Silencer*® Select siRNA (small
30 interfering RNA, Applied Biosystems) directed against *IRF4*, *CRBN*, or *Silencer*® Select Negative Control siRNAs at a final concentration of 0.2–1 μM using Nucleofector® Kit V for

transfection. For IRF4 overexpression, cells were transfected with IRF4- or green fluorescent protein (GFP)- cytomegalovirus (CMV) expression plasmids (OriGene Technologies, Rockville, MD) for 24 h using the Cell Line Nucleofector kit V as above-mentioned. After 24 hours of transfection, cells were treated with lenalidomide for 2 days before luciferase assay, RT-PCR gene expression analysis and Western blot.

Human tumor xenograft model

Female CB17 severe combined immunodeficiency (SCID) mice (6-12 weeks old) were obtained from the Charles River Laboratory (Wilmington, MA) and maintained in microisolator cages under sterile conditions. A total of 10×10^6 OCI-Ly10 DLBCL cells in 100% Matrigel (Becton Dickinson, San Jose, CA) were injected subcutaneously into the right flank of mice. Mice were monitored 2 or 3 times a week for the appearance of tumors. Once the tumors reached an average size of 100 - 150 mg, 10 mice in each group were treated with either vehicle (0.5% carboxymethyl cellulose: 0.25% Tween 80 in deionized H₂O) or indicated doses of lenalidomide (qd x 28, p.o.) or the positive control vincristine (q4d x 4, i.v.). Mice were monitored daily for health status as well as tumor growth. Tumors of all mice were measured with a digital caliper and volumes calculated with the following formula: tumor volume (mm³) = length (mm) X width (mm)². Mice were killed when tumor size exceeded 1000 mm³.

Statistical analysis

Analyses for multiple group comparisons were performed with one-way analysis of variance, followed by Dunnett's post-test, and correlation analyses were carried out using the two-tailed *P*-value Pearson test using GraphPad Prism[®] version 5.01 (San Diego, CA). A value of *P* < .05 was considered significant in all analyses.

Results

6.18.1 ABC-DLBCL cells are more sensitive to lenalidomide than non-ABC-DLBCL cells

In order to define the place of lenalidomide for DLBCL therapy in specific patient populations and to understand the molecular mechanisms of efficacy, a panel of DLBCL cell lines was collected in this study. The DLBCL subtypes of the cell lines were confirmed based on literature information (Lenz G, *et al.*, *Proc Natl Acad Sci U S A* 2008; 105: 13520-5) and molecular analysis, including intracellular NF-κB activity or IRF4 expression, as well as gene expression profiling of key signature genes of activated B cells. See Figures 24A-24C. Cell

proliferation of DLBCL cell lines was found to be inhibited to various degrees by treatment with lenalidomide at a concentration range of 0.01 - 100 μ M. Lenalidomide had minimal effects on proliferation of PBML and GCB-DLBCL cells, but significantly inhibited proliferation of ABC-DLBCL cell lines, except for OCI-Ly3. *See* Figure 24. Lenalidomide treatment also induced apoptosis of sensitive cell lines such as OCI-Ly10. *See* Figure 26.

6.18.2 Lenalidomide reduces IRF4 expression in ABC-DLBCL cells

To understand the molecular mechanism of lenalidomide on ABC-DLBCL cells, the effects of lenalidomide on IRF4 expression in these cells were investigated. Lenalidomide treatment for 1-3 days was found to significantly downregulate IRF4 protein levels in sensitive cell lines such as U2932 and OCI-Ly10, but not the insensitive line OCI-Ly3 cells. *See* Figures 27A-C. Lenalidomide-induced decrease of IRF4 expression occurred as early as 1 day of drug treatment, with similar kinetics to that of the inhibitors (zVRPR-fmk and LY-333,531, respectively) of MALT1 and PKC β , two key enzymes involved in NF- κ B activation upon BCR engagement in B cells. In OCI-Ly3 cells, neither lenalidomide nor the PKC β inhibitor had any appreciable effect on IRF4 levels during 1-3 day treatments. However, the MALT1 inhibitor suppressed IRF4 expression in OCI-Ly3 cells, with complete inhibition observed after 2-3 days of treatment. Taken together, these data suggest that lenalidomide-mediated inhibition of IRF4 expression may be an important mechanism and appears to be related to cell sensitivity to the drug.

6.18.3 Lenalidomide reduces CARD11-BCL-10-MALT1 complex activity of ABC-DLBCL cells

Our DNA sequencing data confirmed previous reports (Lenz, G., *et al.*, *Science* 2008, 319: 1676-9) that the lenalidomide-insensitive ABC-DLBCL line OCI-Ly3 has a unique point mutation in *CARD11* within the exons encoding the coiled-coil domain, while lenalidomide-sensitive ABC-DLBCL lines OCI-Ly10, U2932, TMD8, and Riva do not. *See* Figure 28. This mutation has been reported to cause constitutive formation and activation of CARD11-BCL-10-MALT1 (CBM) complex of BCR signaling pathway, leading to NF- κ B overactivation in lymphoma cells. *See* Lenz, G., *et al.*, *Science* 2008, 319: 1676-9; Thome, M. *et al.*, *Cold Spring Harb Perspect Biol.* 2010; 2: a003004. To investigate the potential involvement of CBM complex in lenalidomide-induced IRF4 inhibition in these cells, the effect of lenalidomide on the complex activity was examined by measuring MALT1 paracaspase enzymatic activity. MALT1

is activated upon association with BCL-10 and CARD11 to form active CBM complex and then cleaves its binding partners, such as BCL-10.. *See* Figures 29A-C.

Similar to the effect of the specific MALT1 inhibitor and PKC β inhibitor, lenalidomide inhibited MALT1-induced BCL-10 cleavage in a concentration-dependent manner in the sensitive ABC-DLBCL cell lines OCI-Ly10 and U2932. Time-kinetic studies revealed that while MALT1- and PKC β -inhibitors affected BCL-10 cleavage within 1 day of treatment, significant inhibition of BCL-10 cleavage by lenalidomide occurred after 2 days of treatment. *See* Figures 29A and 29B. Unlike the MALT1 inhibitor, neither the PKC β inhibitor nor lenalidomide had any effect on BCL-10 cleavage in OCI-Ly3 cells, presumably due to CARD11 mutation which causes over-activation of CBM complex. *See* Figure 29C. These data suggest that lenalidomide, similar to the PKC β inhibitor, can significantly block CBM complex formation/activation or other upstream events in sensitive ABC-DLBCL cells.

6.18.4 Lenalidomide reduces NF- κ B activity of ABC-DLBCL cells but not non-ABC subtype cells

The effect of lenalidomide on NF- κ B activity in various DLBCL cells was examined by measuring the level of NF- κ B subunit proteins binding to consensus sequences and the NF- κ B-driven luciferase activity. As expected, NF- κ B in ABC-DLBCL cells demonstrated increased NF- κ B DNA binding compared to non-ABC-DLBCL. *See* Figure 24B. An NF- κ B-driven luciferase assay demonstrated that lenalidomide inhibited transcriptional activity of NF- κ B by 32-56% in the lenalidomide-sensitive ABC-DLBCL cell lines OCI-Ly10 and U2932 after 2 day drug treatment. *See* Figure 30A. Lenalidomide also partially inhibited DNA binding by Rel A/p65, p50 and c-rel/p70 NF- κ B subunits in a concentration-dependent manner in several ABC-DLBCL cell lines, although the effect was not as potent as the control IKK α / β inhibitor CC-415501. *See* Figures 30B and 30C. In contrast, lenalidomide had no effect on NF- κ B DNA binding in GCB-DLBCL lines nor in normal peripheral blood mononuclear cells. The lenalidomide-insensitive ABC-DLBCL OCI-Ly3 line containing the CARD11 mutation showed significant NF- κ B inhibition only by the IKK α / β inhibitor, and not by lenalidomide. *See* Figures 30B and 30C. These data suggest that lenalidomide inhibits NF- κ B signaling at CBM complex or upstream events in sensitive ABC-DLBCL cells.

6.18.5 Alteration of IRF4 expression in sensitive ABC-DLBCL cells confers resistance to lenalidomide

The dependency role of IRF4 expression in BCR-NF- κ B signal transduction and the sequent effects of lenalidomide treatment upon IRF4 were investigated. Lenalidomide-sensitive ABC-DLBCL cells were transfected with IRF4-specific siRNA or IRF4-CMV-based expression plasmid to modulate IRF4 expression. When U2932 or OCI-Ly10 cells were transfected with IRF4 siRNA to knock down the expression of IRF4, NF- κ B transcriptional activity was diminished by 36-53%. *See* Figure 31A. Thus IRF4 siRNA mimicked the effects of lenalidomide on NF- κ B in these cells. The addition of lenalidomide to the IRF4 siRNA-transfected cells led to even further downregulation of NF- κ B transcriptional activity.

In contrast to IRF4 siRNA transfection, over-expressing IRF4 in these cells for 24 h significantly increased NF- κ B activity by 3.6-7.9 fold. *See* Figure 31B. Furthermore, IRF4 overexpression decreased protein expression levels of the CBM-component BCL-10. *See* Figure 31C. In addition, IRF4 overexpression antagonized the effect of lenalidomide and the PKC β inhibitor but not the IKK inhibitor on NF- κ B -driven luciferase expression in both U2932 and OCI-Ly10 cells. *See* Figure 31D. Therefore, the positive feedback effect of IRF4 on the BCR-NF- κ B pathway appeared to be at a point somewhere between PKC β and IKK.

6.18.6 Cereblon is required for lenalidomide effect on ABC-DLBCL cells

Cereblon has been shown to be a primary mediator of the teratogenicity of thalidomide. Due to its critical role in the anti-myeloma and immunomodulatory activities of lenalidomide and pomalidomide, the role of cereblon in lenalidomide sensitivity of DLBCL was investigated. In ABC-DLBCL cells, knockdown of CRBN with siRNA conferred resistance to lenalidomide as demonstrated by the abrogation of the inhibitory effects of lenalidomide on IRF4 expression, BCL-10 cleavage, NF- κ B activity and proliferation of these cells, while the activity of inhibitors to PKC β and IKK remained unaffected. *See* Figures 32A-32D. These data indicate that antitumor effects of lenalidomide on ABC-DLBCL cells require the presence of cereblon.

6.18.7 Lenalidomide down-regulates IRF4/NF- κ B signaling in OCI-Ly10 mouse xenograft model

To confirm the relevance of lenalidomide-mediated inhibition of NF- κ B/IRF4 signaling *in vivo* in ABC-DLBCL, a subcutaneous OCI-Ly10 xenograft model was established. Lenalidomide at 3-30 mg/kg (po, qdX28) significantly decreased tumor size in this model

($p < 0.01$). *See* Figure 33A. No pronounced toxicity of lenalidomide was observed based upon mouse weight throughout study. Compared with vehicle control group, lenalidomide treatment for 7 days reduced IRF4 expression and BCL-10 cleavage by 15-35% ($p < 0.05$). *See* Figure 33B. These data demonstrate that lenalidomide can reduce IRF4 expression *in vivo* and delays tumor growth in an ABC-DLBCL model, supporting the potential value of lenalidomide as a therapeutic in the treatment of ABC-DLBCL in clinical studies.

6.18.8 IRF4 and CRBN baseline mRNA levels and “ABC scores” of DLBCL cells correlate with sensitivity to lenalidomide

As multiple studies have demonstrated greater lenalidomide sensitivity of ABC-DLBCL cells versus non-ABC-subtypes, potential biomarkers predictive for a therapeutic response to lenalidomide were studied. Pooled *in vitro* data from eleven DLBCL cell lines of various subtypes showed that lenalidomide sensitivity of DLBCL subtypes highly correlated to the baseline level of IRF4 and CRBN mRNA expression. Additionally, the overall “ABC score,” calculated based on baseline levels of signature genes of ABC-DLBCL cells proposed by Staudt, et al. (*Ann. Rev. Med.*, 2002, 53: 303-18), correlated to lenalidomide activity and further confirms the unique sensitivity of this subtype. *See* Figures 34A and 25. Lenalidomide-sensitive ABC-DLBCL cell lines tended to express higher CRBN and IRF4 protein levels than GCB-DLBCL cell lines. *See* Figure 34B. Notably, the lenalidomide-resistant OCI-Ly3 ABC-DLBCL cell line was devoid of CRBN protein expression. These data support the preferential efficacy of lenalidomide in ABC-DLBCL seen in clinical studies, and suggest that the “ABC score,” or the expression of IRF4 or CRBN itself, may serve as potential biomarkers for the prediction of lenalidomide efficacy.

6.19 Polyclonal CRBN70 antibody

Rabbit polyclonal antibody CRBN70 was generated by inoculating rabbits with the CRBN peptide sequence EEFHGRTLHDDDD (SEQ ID:1), wherein a C-terminal cysteine EEFHGRTLHDDDDC (underlined) is further used to couple the peptide to Keyhole Limpet Hemocyanin (KLH).

Seq.	Mod.
EEFHGRTLHDDDDC-KLH	KLH-Peptide Cys

The peptide depicted in SEQ ID NO:1 used to make the antibody corresponds to amino acids 65-76 (bolded) of CRBN isoform 1 (NP_057386) (SEQ ID NO:12):

```

5      1 magegdqgda ahnmgnhlpl lpaeseede mevedqdske akkpniinf dtslptshtyl
      61 gadmeefhgr tlhdddscqv ipvlpqvmml lipgqtlplq lfhpqevsmv rnliqkdrtf
      121 avlaysnvqe reaqfgttae iyayreeqdf gieivkvkai grqrfkvlel rtqsdgiqqa
      181 kvqilpecvl pstmsavqle slnkcfifps kpvsredqcs ykwwqkyqkr kfhcanltsw
      241 prwlyslyda etlmdrikkq lrewdenlkd dslpsnpidf syrvaacdpi ddvlriqlk
      301 igsaiqrllrc eldimnkcts lcckqcqete ittkneifsl slcgpmayyv nphgyvhetl
10     361 tvyakcnlnl igrpstehsw fpgyawtvaq ckicashigw kftatkkdms pqkfwgltrs
      421 allptipdte deispdkvil cl

```

It is noted that isoform 2 of CRBN (GenBank Accession No. NP_001166953; SEQ ID NO:13) uses an alternate in frame splice site that results in the elimination of the alanine (underlined), but has no other changes:

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      1 magegdqgda ahnmgnhlpl lpeseeedem evedqdskea kkpniinf dtslptshtylg
      61 admeefhgrt lhdddscqvi pvlpqvmml ipgqtlplql fhqpqevsmv rnliqkdrtf
      121 vlaysnvqe eaqfgttaei yayreeqdfg ieivkvkaig rqrqfkvlel rtqsdgiqqak
      181 vqilpecvlp stmsavqles lnkcqifpsk pvsredqcsy kwwqkyqkrk fhcanltswp
20     241 rwlyslydae tlm drikkql rewdenlkdd slpsnpidfs yrvaacldpid dvlriqlkki
      301 gsaiqrllrce ldimnkctsl cckqcqetei ttkneifsls lcgpmayyv nphgyvhetlt
      361 vykacnlnli grpstehswf pgyawtvaqc kicashigwk ftatkkdmsp qkfwgltrsa
      421 llptipdted eispdkvilc l

```

The polyclonal CRBN70 antibody was purified. Purified antibody was then titered by indirect ELISA against the peptide or protein bound to a solid-phase to measure the reactivity of the antibodies after elution and the amount of antibody remaining in serum (the flow-through).

Sample	Volume (mL)	Conc. (mg/mL)	Total (mg)	Titer (ng)	Flow- through
CRBN70	6	0.414	2.484	5	2048

The eluent “titer” indicates the minimum concentration at which the CRBN70 antibody can effectively detect the CRBN antigen. The “flow-through” titer represents the reactivity of the antibodies remaining in the serum after it has been passed through the column.

6.20 Sequences of VH and VL of anti-CRBN antibodies

Rabbits were primed with amino acid sequence 65-76 (SEQ ID NO:1) of human CRBN (SEQ ID NO:12), and the spleen was removed for IgG subtyping and monoclonal antibody creation.

The IgG heavy and light chains from two CGN-6 hybridoma clones were sequenced by Epitomics. The two clones were CGN-6-1-11 and CGN-6-4-5.

Brief methods description of RabMAb IgG molecular cloning:

Messenger RNA (mRNA) from hybridoma cells was isolated using TURBOCAPTURE Kit (Qiagen: Catalog #72232) following the manufacturer's suggested protocol, and then reverse transcribed into cDNA using oligo-dT primer. The variable region of heavy chain (VH) was PCR amplified using proprietary primers OYZ64-2 and OYZvh3. The entire light chain (LC) was PCR amplified using proprietary primers OYZ62 and OYZ71. The PCR products were resolved on 1% argrose gel followed by purification using Qiagen gel purification kit (Qiagen: Catalog #28704), and the purified DNA fragments were subjected to sequencing.

CGN-6-1-11- Heavy chain nucleotide sequence (SEQ ID NO:4):

ATGGAGACTGGGCTGCGCTGGCTTCTCCTGGTCGCTGTGCTCAAAGGTGTCCACTGTCACTGAGTGGAGGAGTCCGG
GGGTGCGCTGGTCACGCCTGGGACACCCCTGACACTCACCTGTACAGTCTCTGGATTCTCCCTCAGTTACTATGGAG
TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTAGAATACATCGGATACATTTATAGTGATAGTGATAAGACATAC
TACGCGACCTGGGCGAAAGGCCGATTACCATCTCCAAAACCTCGACCACGGTGGATTGTGAAAATCACCAGTCCGAC
AATCGAGGACACGGCCACCTATTTCTGTGCCAGAGGTAATCCGCTTGTAGTTATAGCATCTGGGGCCCAGGCACCC
TGGTCACCGTCTCCTTAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACCCAGC
TCCACGGTGACCTGGGCTGCCTGGTCAAAGGTACCTCCCGGAGCCAGTGACCGTGACCTGGAACCTCGGGCACCT
CACCAATGGGGTACGCACCTTCCCGTCCGTCCGGCAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGA
CCTCAAGCAGCCAGCCCGTCACCTGCAACGTGGCCACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCGCCC
TCGACATGCAGCAAGCCACGTGCCCCACCCCTGAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCCAAAACC
CAAGGACACCTCATGATCTCACGCACCCCGAGGTACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCGAGG
TGCAGTTCACATGGTACATAAACAACGAGCAGGTGCGCACCGCCCGCCGCTACGGGAGCAGCAGTTCAACAGC
ACGATCCGCGTGGTCAGCACCTCCCCATCGCGCACCAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCA
CAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCCAGAGGGCAGCCCTGGAGCCGAAGGTCTACA
CCATGGGGCCCTCCCGGGGAGGAGCTGAGCAGCAGGTGCGTCAGCCTGACCTGCATGATCAACGGCTTCTACCCCTCC
GACATCTCGGTGGAGTGGGAGAAGAAGCGGAAGGCAGAGGACAACATAAGACCACGCCGGCCGTGCTGGACAGCGA
CGGCTCCTACTTCTCTACAGCAAGCTCTCAGTGCCACGAGTGAGTGGCAGCGGGGCGACGTCTTACCTGCTCCG
TGATGCACGAGGCCTTGACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA

CGN-6-1-11- Heavy chain protein sequence (SEQ ID NO:5):

METGLRWLLLVAVLKGVCQSVESGGRLVTPGTPLTLTCTVSGFSLSYGVSWVRQAPG
KGLYIGYIYSDSKTYATWAKGRFTISKSTTTVDLKITSPITIEDTATYFCARGTPLAS
YSIWGPGLVTVSLGQPKAPSVFPLAPCCGDTSPSTVTLGCLVKGYLPEPVTVTWSNGL
TNGVRTFSPVRQSSGLYSLSSVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCP
PPELLGGPSVFI FPPKPKDTLMISRTPEVTCVVDVDSQDDPEVQFTWYINNEQVRTARPP
LREQQFNSTIRVSTLPIAHQDWLRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVYT
MGPPREELSSRSVSLTCMINGFYPSDISVEWEKNGKAEDNYKTTPAVLDSGYSFLYSKL
SVPTSEWQRGDVFTCSVMHEALHNHYTQKSISRSPGK-

CGN-6-1-11- Light chain nucleotide sequence (SEQ ID NO:6):

ATGGACACGAGGGCCCCCACTCAGCTGCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCACATTTGCCAGGTGCT
GACCCAGACTCCAGCCTCGGTGTCTGCAGCTGTGGGAGGCACAGTCACCATCAATTGCCAGGCCAGTCAGAGTGTTT
ATAAGAATAACTATTTATCCTGGTTTCAGCAGAAACCAGGGCAGCCTCCCAAGCTCCTGATTTACGAAGCGTCCAAA
CTGGCATCTGGGGTCCCCCGCGTTCAAAGGCAGTGGATTGGGACACAGTTCACTTTACCATTAGCGACCTGGA
GTGTGACGATGCTGCCTTTTACTACTGTGCAGGCGTTATTATGGTAATATTTTTTTTTTCGGCGGAGGGACCGAGG
TGGTGGTCAAAGGTGATCCAGTTGCACCTACTGTCTCATCTTCCACCAGCTGCTGATCAGGTGGCAACTGGAACA

GTCACCATCGTGTGTGTGGCGAATAAATACTTTCCCGATGTCACCGTCACCTGGGAGGTGGATGGCACCACCCAAAC
 AACTGGCATCGAGAACAGTAAACACCGCAGAATTCTGCAGATTGTACCTACAACCTCAGCAGCACTCTGACACTGA
 CCAGCACACAGTACAACAGCCACAAAGAGTACACCTGCAAGGTGACCCAGGGCACGACCTCAGTCGTCCAGAGCTTC
 AATAGGGGTGACTGTTAG

5 CGN-6-1-11- Light chain protein sequence (SEQ ID NO:7):

MDTRAPTQLLGLLLLWLPGATFAQVLTQTTPASVSAAVGGTVTINCQASQSVYKNNYLSWF
 QQKPGQPPKLLIYEASKLASGVPPRFKGSFGTQFTFTISDLECDAAFYYCAGGYGNI
 FFFGGGTEVVVKGDPVAPTVLIFPPAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQT
 TGIENSKTPQNSADCTYNLSSTLTLTSTQYNHKEYTCKVTQGTTSVVQSFNRGDC-

10

CGN-6-4-5- Heavy chain nucleotide sequence (SEQ ID NO:8):

ATGGAGACTGGGCTGCGCTGGCTTCTCCTGGTCGCTGTGCTCAAAGGTGTCCAGTGTGAGTCGGTGGAGGAGTCCGG
 GGGTCGCCTGGTCACGCCTGGGACACCCCTGACACTCACCTGCACAGTCTCTGGATTCTCCCTCAGTAGGTATGGAG
 TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGCTGGAACACATCGGATACATTTATAGTGATCCTGGTATGACATTC
 15 TACGCGACCTGGGCGAAAGGCCGATTACCATCTCCAAAACCTCGTCGACCACGGTGGATTTGAAAATGACCAGTCC
 GACAATCGAGGACACGGCCACCTATTTCTGTGCCAGAGGTACTCCGCTTGCTAGTTATAGCACCTGGGGCCAGGCA
 CCCTGGTCACCATCTCCTTAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACCC
 AGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGGTACCTCCCGGAGCCAGTGACCGTGACCTGGAACCTCGGGCAC
 CCTCACCAATGGGGTACGCACCTTCCCGTCCGTCCGGCAGTCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCG
 20 TGACCTCAAGCAGCCAGCCCGTCACCTGCAACGTGGCCACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCG
 CCCTCGACATGCAGCAAGCCACGTGCCACCCCTGAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCC
 ACCCAAGGACACCCCTCATGATCTCACGCACCCCGAGGTACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCG
 AGGTGCAGTTCACATGGTACATAAACAACGAGCAGGTGCGCACCCGCGCCGCGCTACGGGAGCAGCAGTTCAAC
 AGCACGATCCGCGTGGTCAGCACCCCTCCCCATCGCGCACCCAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGT
 25 CCACAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCCAGAGGGCAGCCCCCTGGAGCCGAAGGTCT
 ACACCATGGGCCCTCCCGGGAGGAGCTGAGCAGCAGGTGCGTCAGCCTGACCTGCATGATCAACGGCTTCTACCCCT
 TCCGACATCTCGGTGGAGTGGGAGAAGAACGGGAAGGCAGAGGACAACCTACAAGACCACGCGCGCGTGTGAGACAG
 CGACGGCTCCTACTTCTCTACAGCAAGCTCTCAGTGCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCT
 CCGTGATGCACGAGGCCTTGCACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA

30 CGN-6-4-5- Heavy chain protein sequence (450 amino acids) (SEQ ID NO:9):

METGLRWLLLVAVLKGVCQSVEESGGRLVTPGTPLTLTCTVSGFSLSRYGVSWSVRQAPG
 KGLEHIGYIYSDPGMTFYATWAKGRFTISKTSSTTVDLKMTSPTIEDTATYFCARGTPLA
 SYSTWGPGLVTISLGQPKAPSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGT
 LTNGVRTFPSVRQSSGLYSLSSVSVTSSSQPVTCNVAPATNTKVDKTVAPSTCSKPTC
 35 PPELLGGPSVFIFFPKPKDTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVVRTAR
 PLREQQFNSTIRVVSTLPIAHQDWLRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVY
 TMGPPREELSSRSVSLTCMINGFYPSDISVEWEKNGKAEDNYKTTPAVLDSGYSYFLYSK
 LSVPTSEWQRGDFVFTCSVMHEALHNHYTQKSISRSPGK-

CGN-6-4-5- Light chain nucleotide sequence (SEQ ID NO:10):

ATGGACACGAGGGCCCCCACTCAGCTGCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCACATTTGCTCAAGTGCT
 GACCCAGACTCCAGCCTCCGTGTCTGCAGCTGTGGGAGGCACAGTCACCATCAATTGCCAGTCCAGTGAGAATATTT
 5 ATAAGAACAACACTACTTATCCTGGTTTCAGCAGAAACCAGGGCAGCCTCCCAAGCTCCTGATCTATCAGGCATCCACT
 CTGGCATCTGGGGTCCCATCGCGGTTCAAAGGCAGTGGATCTGGGACACGATTCAGTCTCACCATCAGCGACCTGGA
 GTGTGACGATGCTGCCACTTACTACTGTGCAGGCGGTTATAGTGGTAATATTTTTACTTTTCGGCGGAGGGACCGAGG
 TGGTGGTCAAAGGTGATCCAGTTGCACCTACTGTCTCATCTTCCCACCAGCTGCTGATCAGGTGGCAACTGGAACA
 GTCACCATCGTGTGTGTGGCGAATAAATACTTTCCCGATGTCACCGTCACCTGGGAGGTGGATGGCACCACCCAAAC
 10 AACTGGCATCGAGAACAGTAAACACCGCAGAATTCTGCAGATTGTACCTACAACCTCAGCAGCACTCTGACACTGA
 CCAGCACACAGTACAACAGCCACAAAGAGTACACCTGCAAGGTGACCCAGGGCACGACCTCAGTCGTCCAGAGCTTC
 AATAGGGGTGACTGTTAG

CGN-6-4-5- Light chain protein sequence (SEQ ID NO:11):

MDTRAPTQLLGLLLLWLPATFAQVLTQTPASVSAVGGTVTINCQSSSENIYKNNYLSWF
 QQKPGQPPKLLIYQASTLASGVPSRFKGS GSGTRFSLTISDLECDDAATYYCAGGYSGNI
 15 FTFGGGTEVVVKGDPVAPTFLIFPPAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQT
 TGIENSKTPQNSADCTYNLSSTLTLTSTQYNHKEYTCKVTQGTTSVVQSFNRGDC-

Amino acid sequence alignments of the heavy and light chains of the CGN6-1-11 and CGN 6-4-5 antibodies are provided in Figures 35 (heavy chains) and 36 (light chains).

6.21 Immunoblot and immunofluorescence with monoclonal anti-CRBN antibody, CGN-6-4-5

Rabbit monoclonal antibody CGN-6-4-5 specifically recognizes the full-length 51 kDa human CRBN protein on denaturing immunoblots.

Confocal microscopic Immunofluorescence:

CGN-6-4-5 was diluted 1:1000. Exemplary cell staining of DF15 and DF15R cells is shown in Figure 37. In particular, Figures 37A and 37B depict confocal immunofluorescent analysis of DF15 (left panel) and DF15R cells (right panel) using 1 µg/ml CGN-6-4-5 antibody (green) (A) or CGN-6-4-5 antibody / CRBN blocking peptide mix (1:5 excess ratio) (B). Nuclear staining was performed with Dapi (blue).

Immunoblot

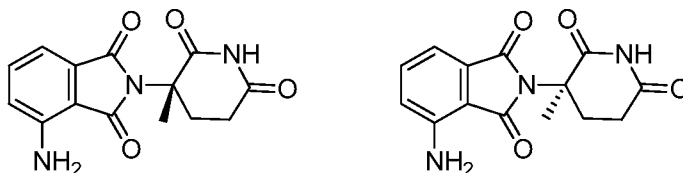
CGN-6-4-5 was diluted 1: 10,000 dilution in 0.1% Tween PBS buffer. An exemplary immunoblot with myeloma cells containing endogenous CRBN (DF15), DF15R with no CRBN and HEK293 cells expressing recombinant flag-tagged CRBN is shown in Figure 38. Peptide neutralization was performed by combining antibody with a five-fold (by weight) excess of blocking peptide in 500 µl PBS and incubating with constant rotation at room temperature for 2 hours.

6.22 Thalidomide, lenalidomide and pomalidomide bind to CRBN via the glutarimide moiety

The binding of phthalimide and glutarimide to CRBN was investigated in order to elucidate the mechanism of CRBN binding of thalidomide, lenalidomide, pomalidomide and structurally similar compounds. Glutarimide bound to CRBN while phthalimide did not. Thus, these results support the hypothesis that thalidomide, lenalidomide and pomalidomide bind to CRBN via the glutarimide moiety. See Figure 39A.

6.23 CRBN binding of methyl-pomalidomide is enantioselective

The binding of S-methyl-pomalidomide and R-methyl-pomalidomide was investigated in order to determine whether CRBN binding is enantioselective. S-methyl-pomalidomide had greater affinity for CRBN than R-methyl-pomalidomide. See Figure 39B.



S-methyl-pomalidomide

R-methyl-pomalidomide

The examples set forth above are provided to give those of ordinary skill in the art with a complete disclosure and description of how to make and use the claimed embodiments, and are not intended to limit the scope of what is disclosed herein. Modifications that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All publications, patents, and patent applications cited in this specification are incorporated herein by reference as if each such publication, patent or patent application were specifically and individually indicated to be incorporated herein by reference.

What is claimed is:

1. A method of selecting a group of cancer patients based on the level of CRBN expression, or the levels of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 or NFκB expression within the cancer, for the purposes of
5 predicting clinical response, monitoring clinical response, or monitoring patient compliance to dosing by thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof; wherein the cancer patients are multiple myeloma, non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, melanoma or
10 solid tumor patients.
2. The method of claim 1, wherein the cancer patients are multiple myeloma patients.
3. The method of claim 1, wherein the cancer patients are non-Hodgkin's lymphoma patients.
4. The method of claim 1, wherein the method of selecting a group of cancer
15 patients is based on the level of DDB1 expression within the cancer.
5. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of DDB2 expression within the cancer.
6. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of GSK3B expression within the cancer.
- 20 7. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of CUL4A expression within the cancer.
8. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of CUL4B expression within the cancer.
9. The method of claim 1, wherein the method of selecting a group of cancer
25 patients is based on the level of XBP-1 expression within the cancer.
10. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of FAS1 expression within the cancer.

11. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of RANBP6 expression within the cancer.

12. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of DUS3L expression within the cancer.

5 13. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of PHGDH expression within the cancer.

14. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of AMPK expression within the cancer.

10 15. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of IRF4 expression within the cancer.

16. The method of claim 1, wherein the method of selecting a group of cancer patients is based on the level of NFκB expression within the cancer.

15 17. A method of identifying or monitoring multiple myeloma patient resistance to thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione therapy, based on the presence or appearance of mutations within a CRBN gene.

18. The method of claim 17, wherein the mutation with the CRBN gene is a single-nucleotide polymorphism in the coding region c.745C>CA causing an amino acid change 249D>YD in the protein within the DDB1 binding domain of CRBN.

20 19. A method of selecting a group of cancer patients responsive to treatment with thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof;

25 based on the level of CRBN expression, or the levels of DDB1, DDB2, GSK3B, CUL4A, CUL4B, XBP-1, FAS1, RANBP6, DUS3L, PHGDH, AMPK, IRF4 or NFκB expression within the patient's T cells, B cells, or plasma cells, for the purposes of predicting clinical response, monitoring clinical response, or monitoring patient compliance to dosing by thalidomide, lenalidomide, pomalidomide or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-

dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

20. The method of claim 19, wherein the cancer patients are multiple myeloma, non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, melanoma or solid tumor patients.

5 21. The method of claim 20, wherein the cancer patients are multiple myeloma patients.

22. The method of claim 20, wherein the cancer patients are diffuse large B-cell lymphoma patients.

10 23. The method of claim 22, wherein the diffuse large B-cell lymphoma is of the activated B-cell-like subtype.

24. The method of claim 21, wherein the method of selecting the group of cancer patients is based on the level of CRBN or IRF4 expression within the patient's T cells, B cells, or plasma cells.

15 25. An isolated antibody that immunospecifically binds to an epitope in CRBN, wherein the epitope has the amino acid sequence SEQ ID NO:1.

26. The isolated antibody of claim 25, wherein the antibody is polyclonal.

27. An antibody that immunospecifically binds CRBN, wherein the antibody comprises: a heavy chain having the amino acid sequence depicted in SEQ ID NO:5 or 9.

20 28. An antibody that immunospecifically binds CRBN, wherein the antibody comprises a light chain having the amino acid sequence depicted in SEQ ID NO:7 or 11.

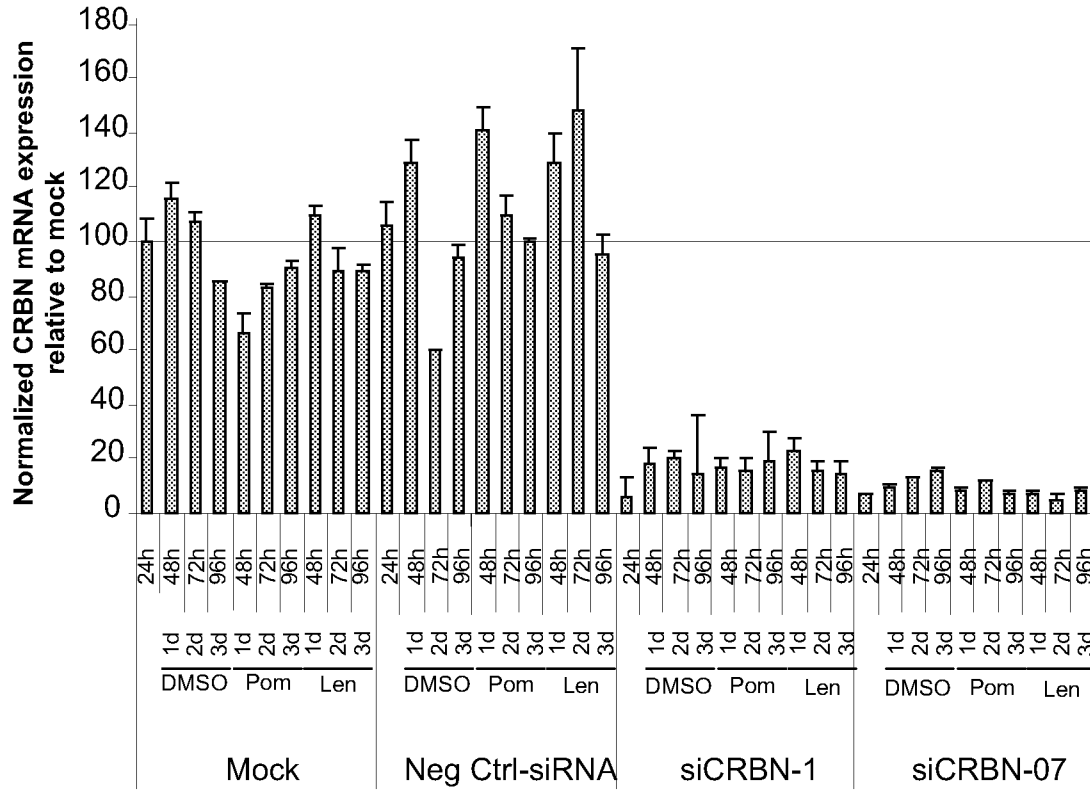
29. An isolated antibody that comprises a heavy chain having the amino acid sequence depicted in SEQ ID NO:5 and a light chain having the amino acid sequence depicted in SEQ ID NO:7.

25 30. An isolated antibody, comprising a heavy chain having the amino acid sequence depicted in SEQ ID NO:9 and a light chain having the amino acid sequence depicted in SEQ ID NO:11.

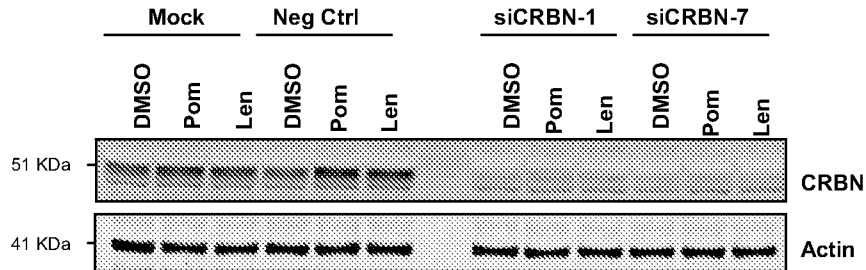
31. A method of utilizing the antibody according to claim 25 to measure expression levels of CRBN in patient tumor or host cells, to predict clinical response, monitor clinical response, monitor patient compliance to dosing, or monitor development of resistance to therapy with thalidomide, lenalidomide, pomalidomide, or 3-(5-amino-2-methyl-4-oxo-4*H*-quinazolin-3-yl)-piperidine-2,6-dione, a stereoisomer thereof, or a pharmaceutically acceptable salt, solvate, hydrate, co-crystal, clathrate, or polymorph thereof.

FIG. 1A & 1B

A



B



C

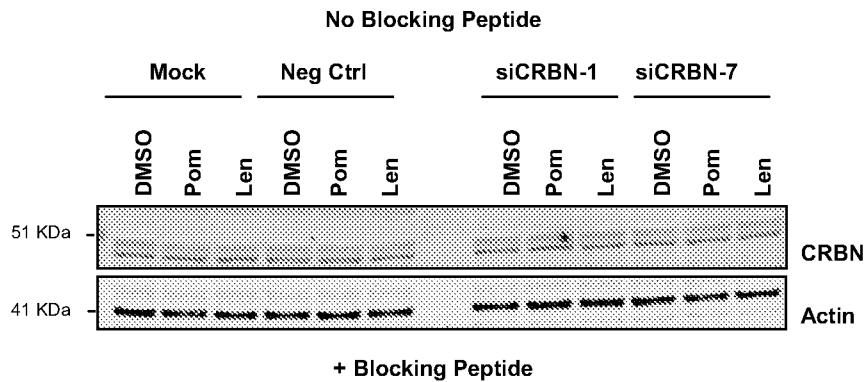


FIG. 2A & 2B

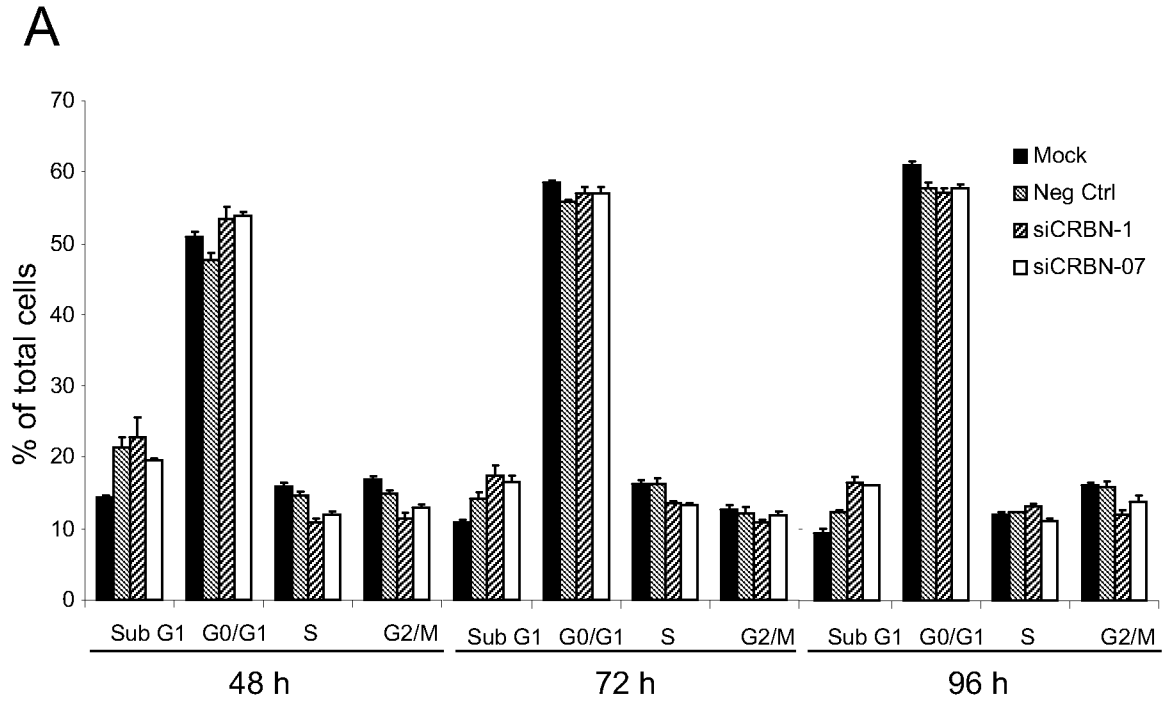
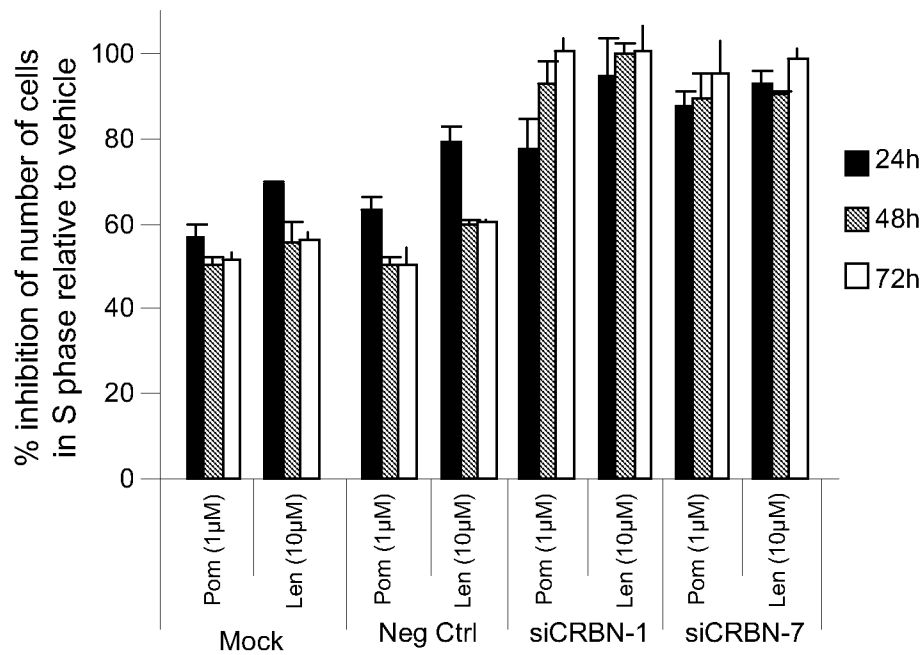
**B**

FIG. 2C

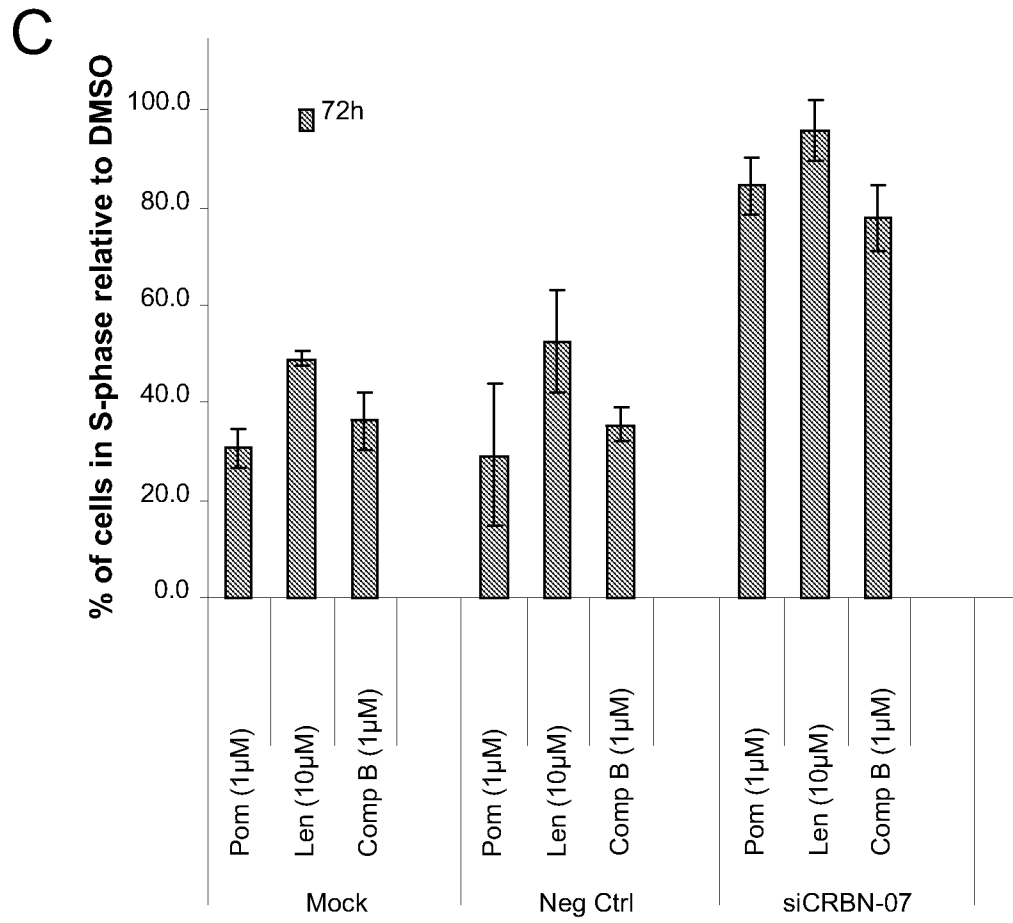


FIG. 3A & 3B

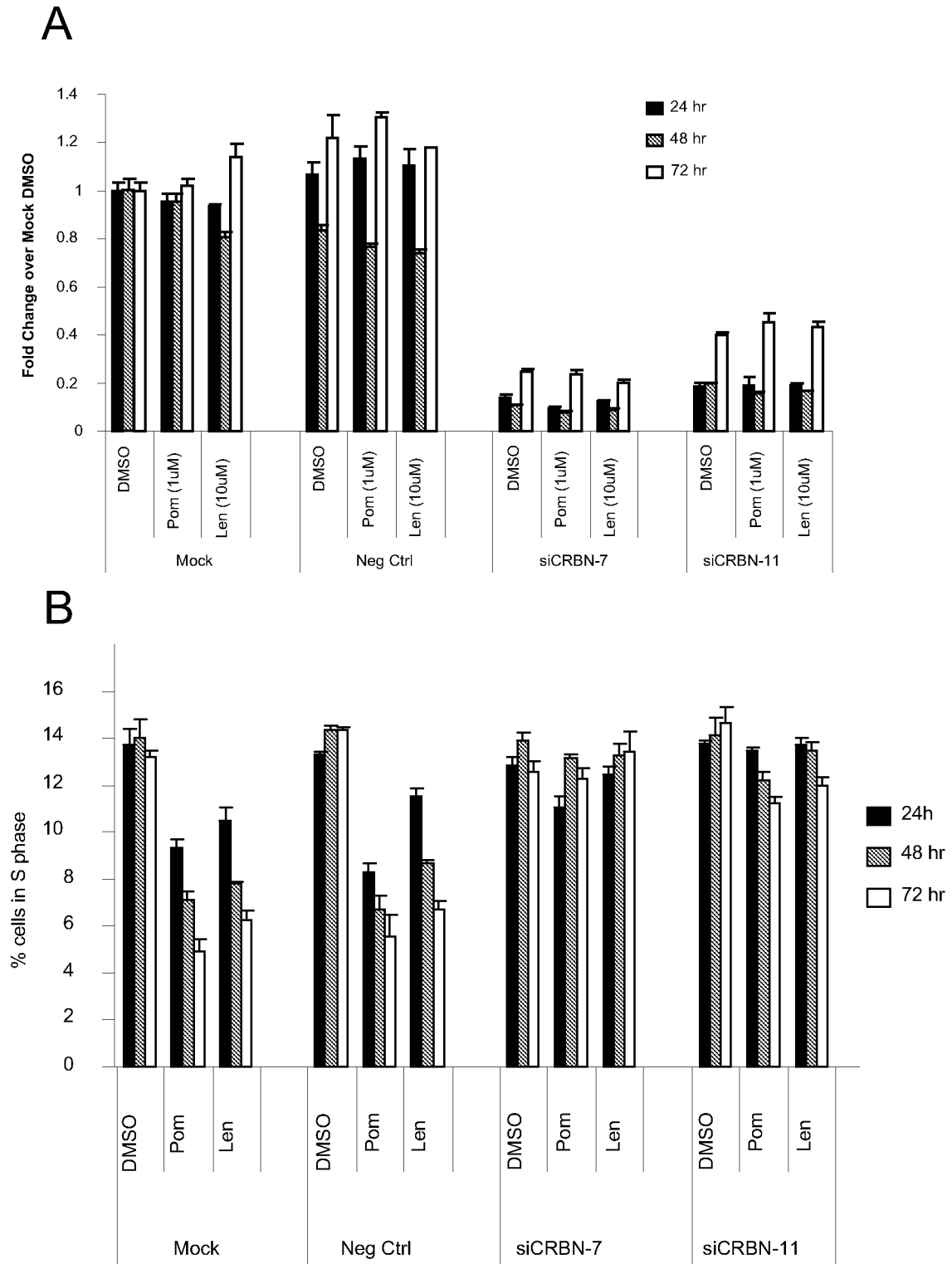
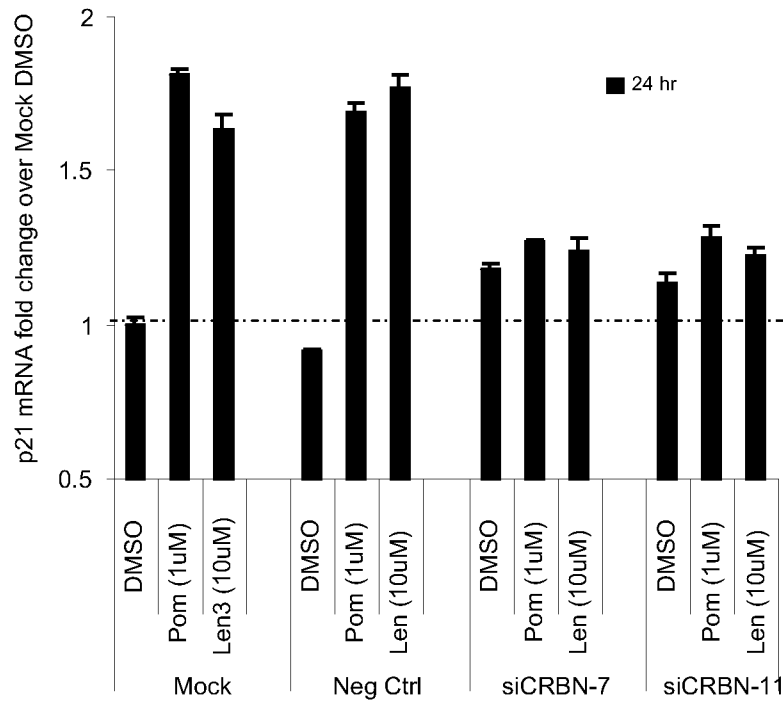
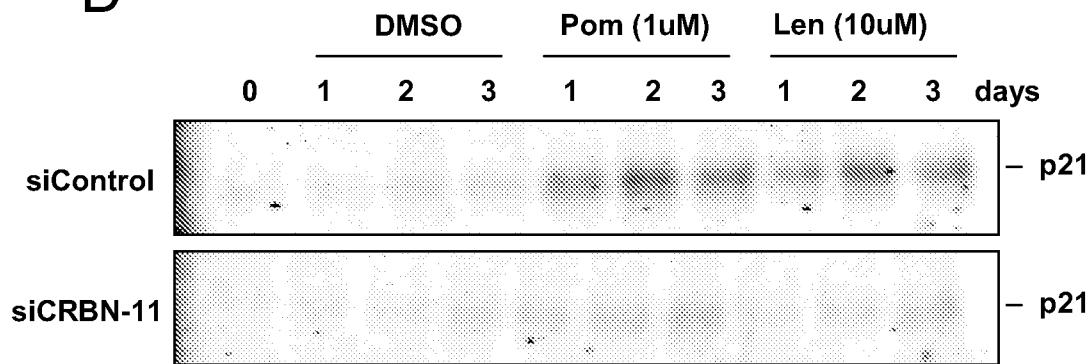


FIG 3C & 3D

C



D



p21 western blot

FIG 4A & 4B

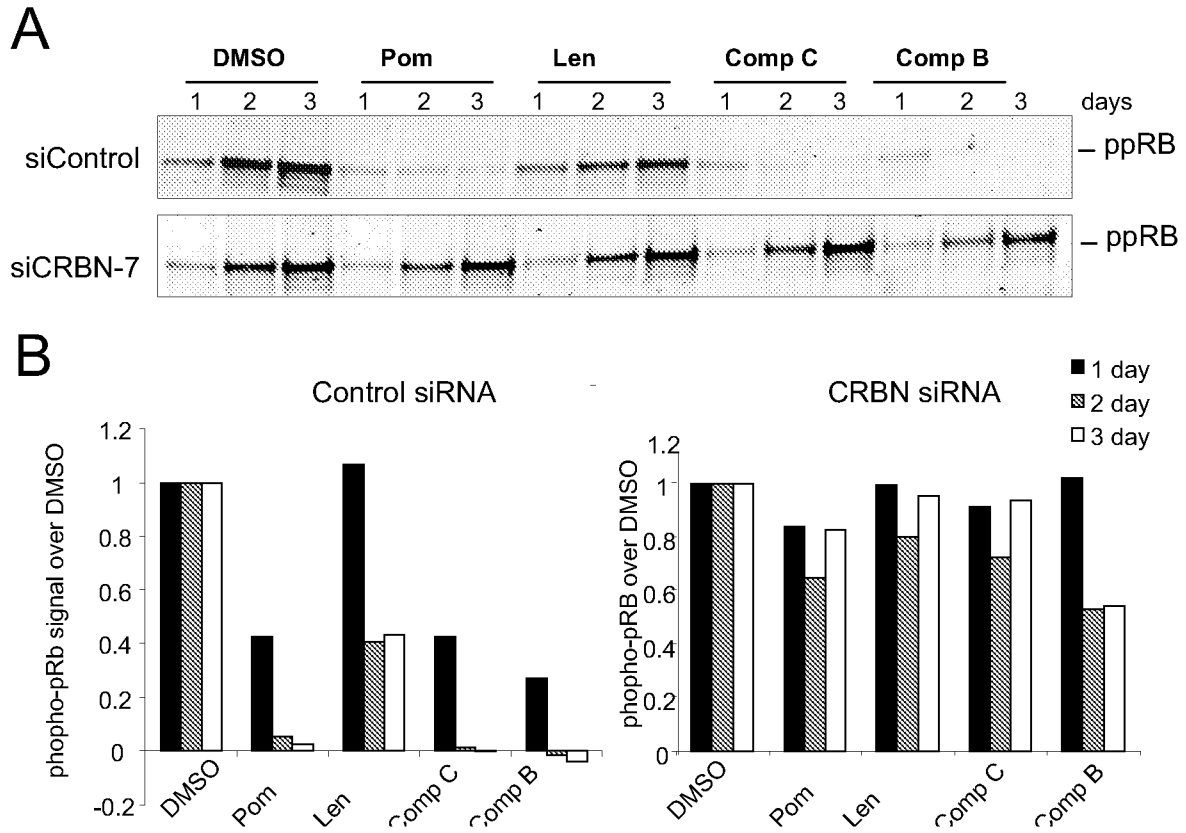


FIG 4C & 4D

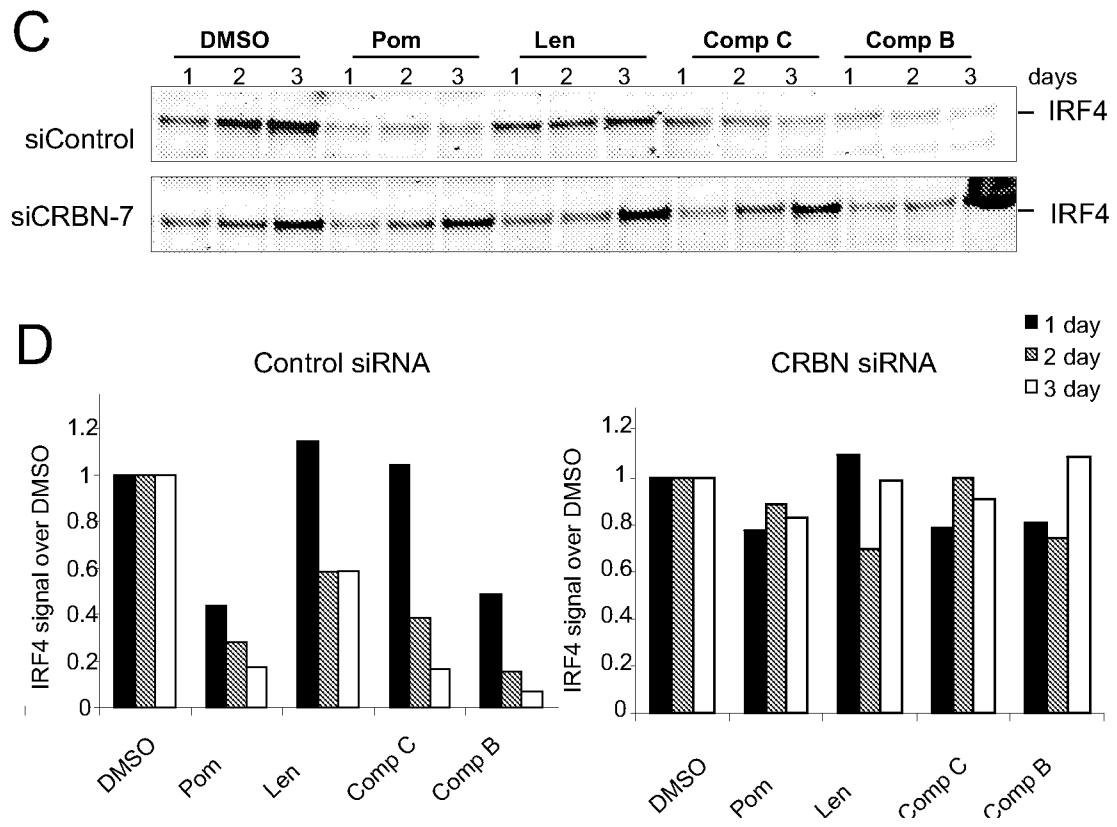


FIG 5A & 5B

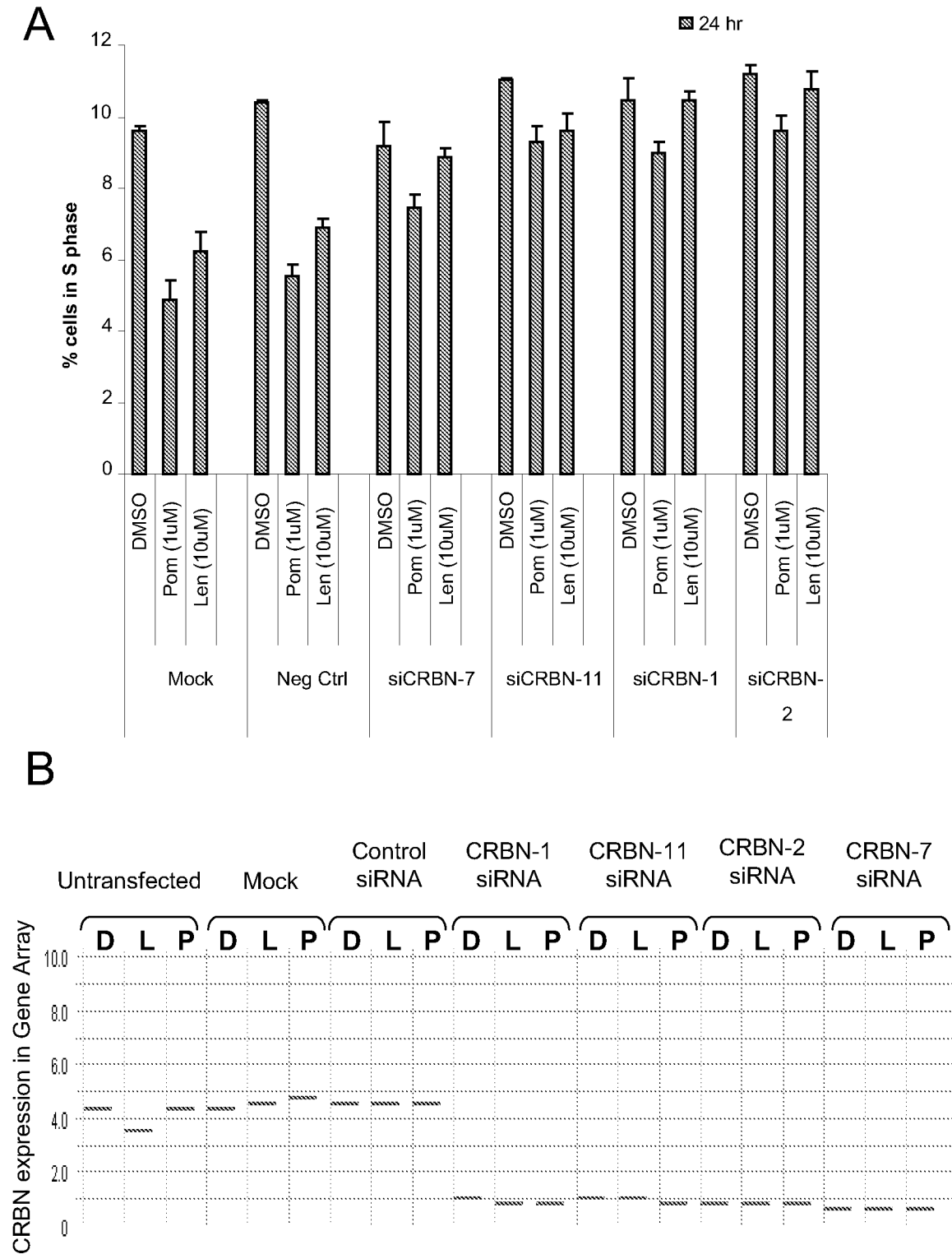


FIG 5C

C

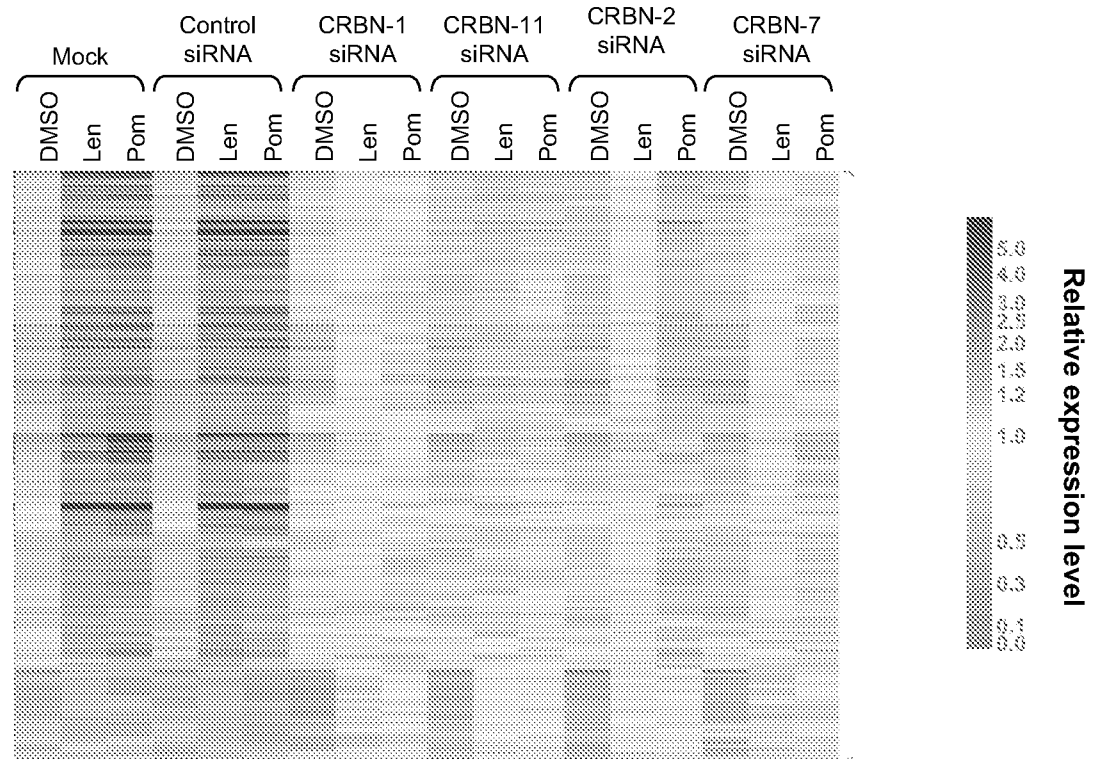
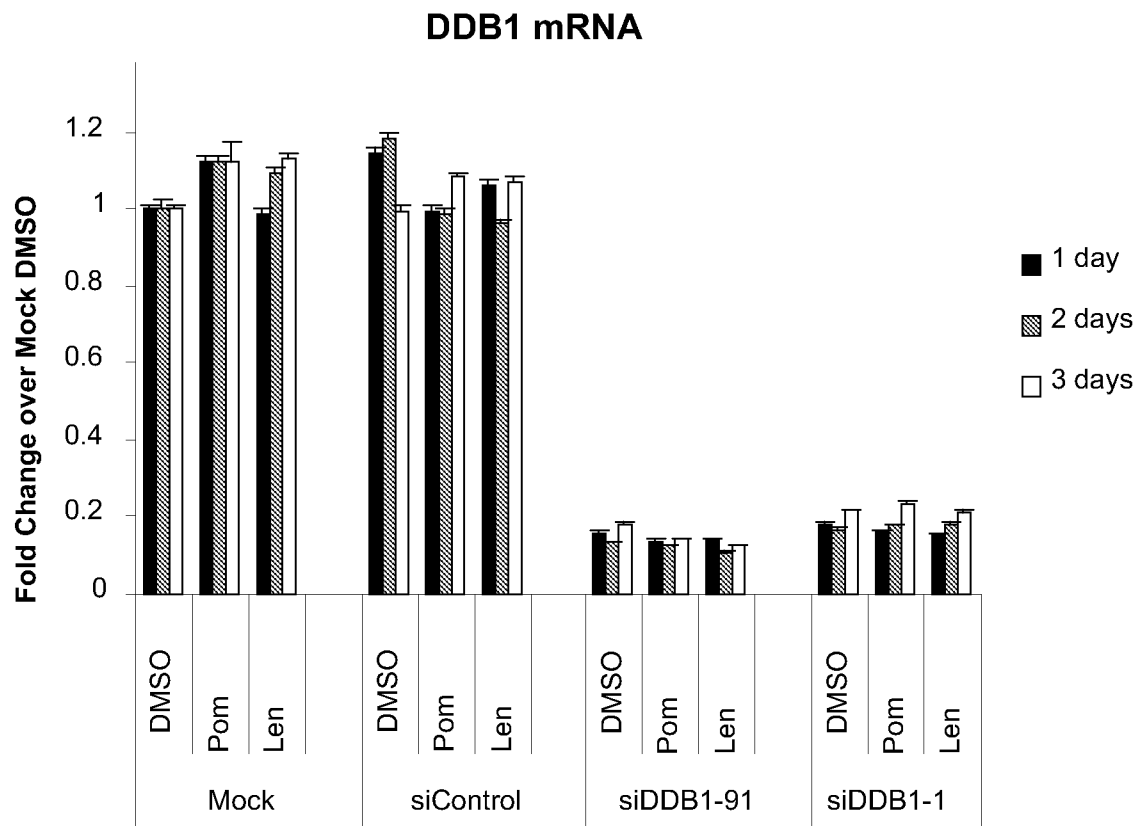


FIG 6A & 6B

A



B

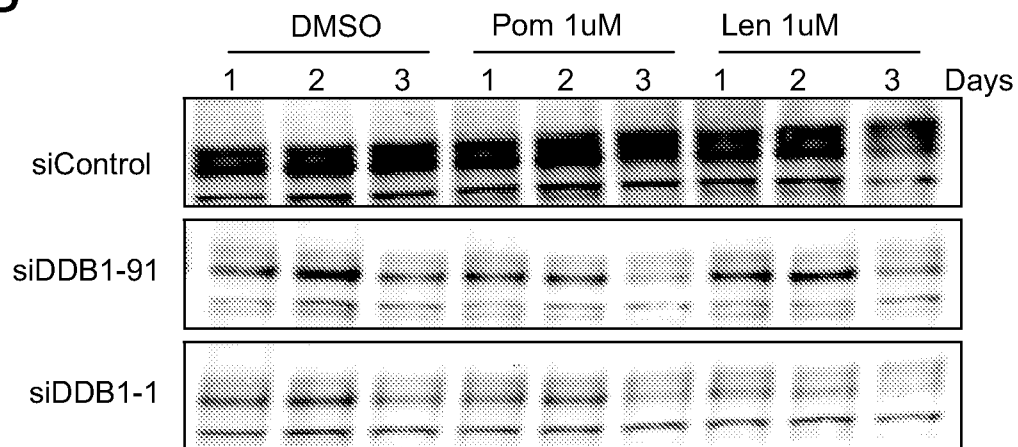


FIG 6C

C

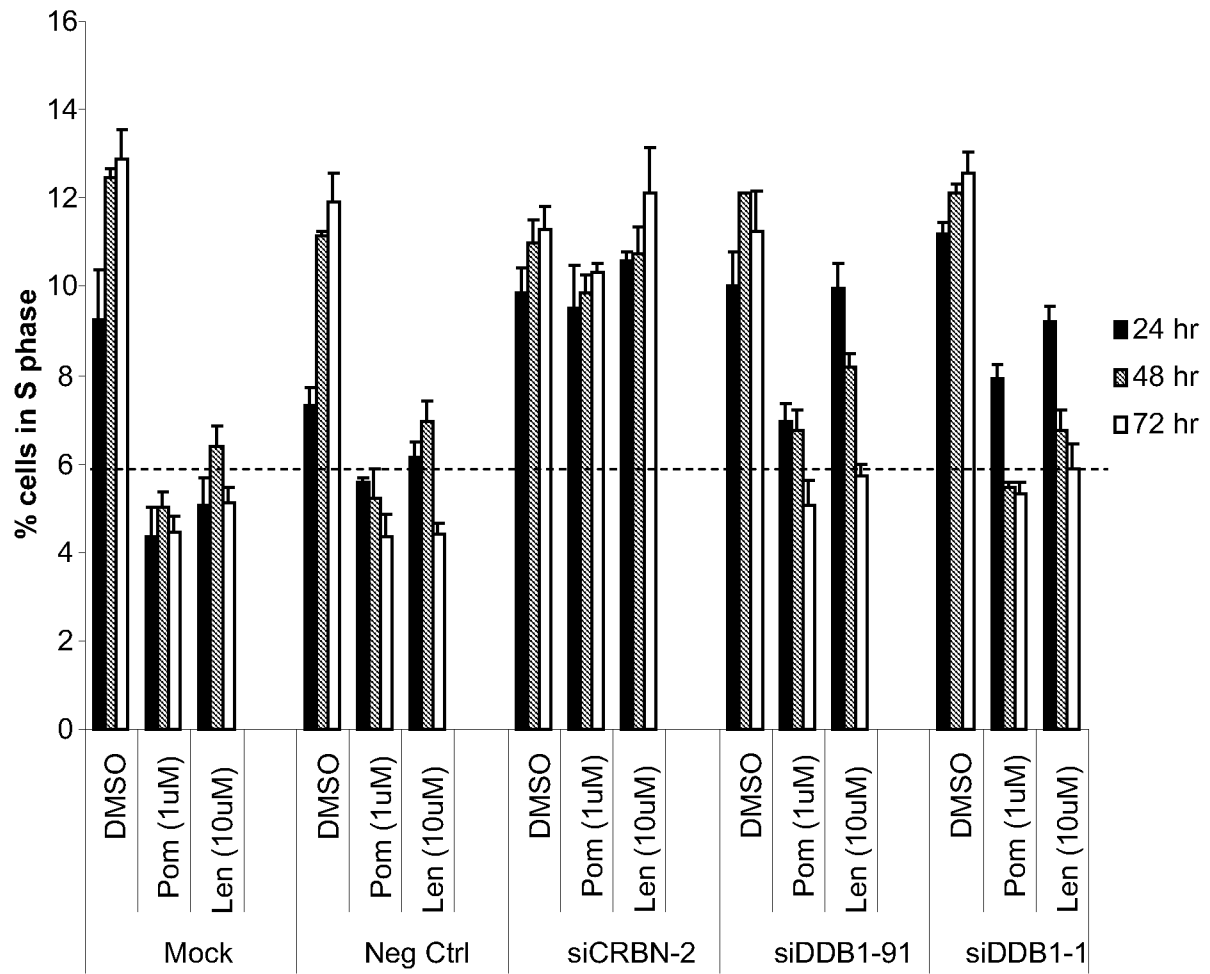


FIG 6D

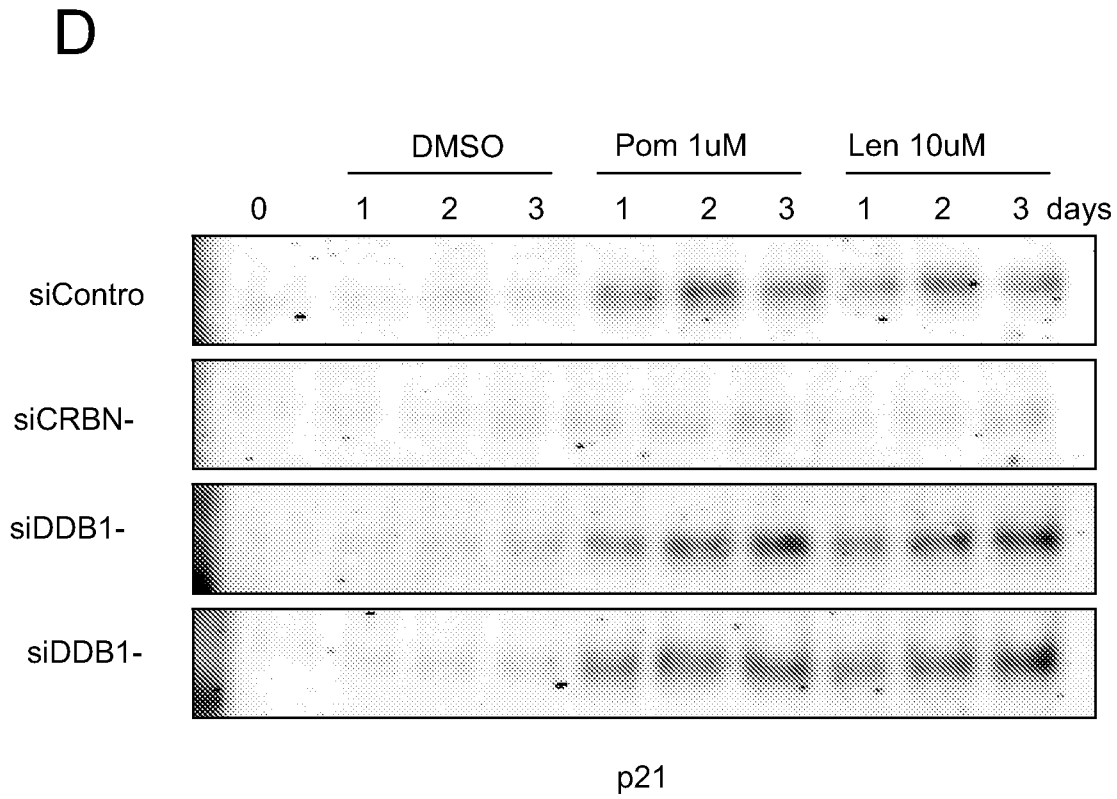


FIG 7A

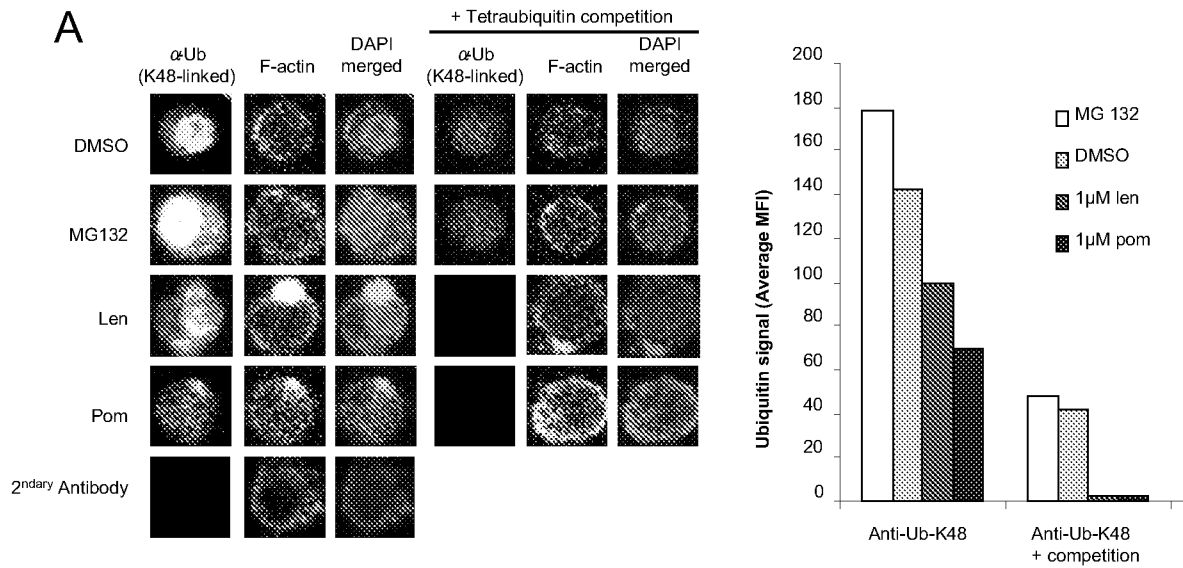
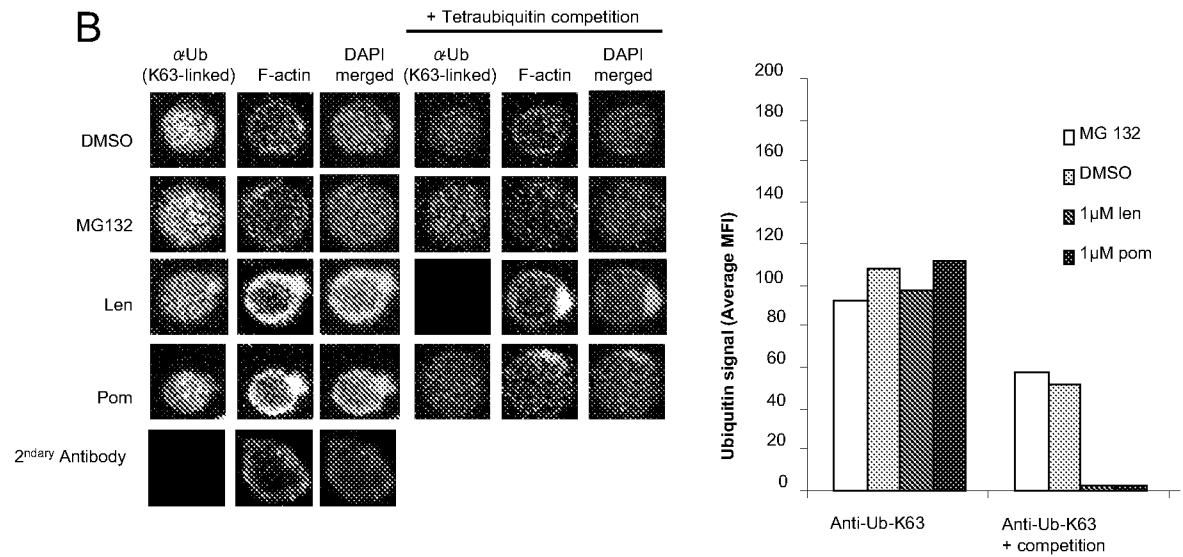


FIG 7B



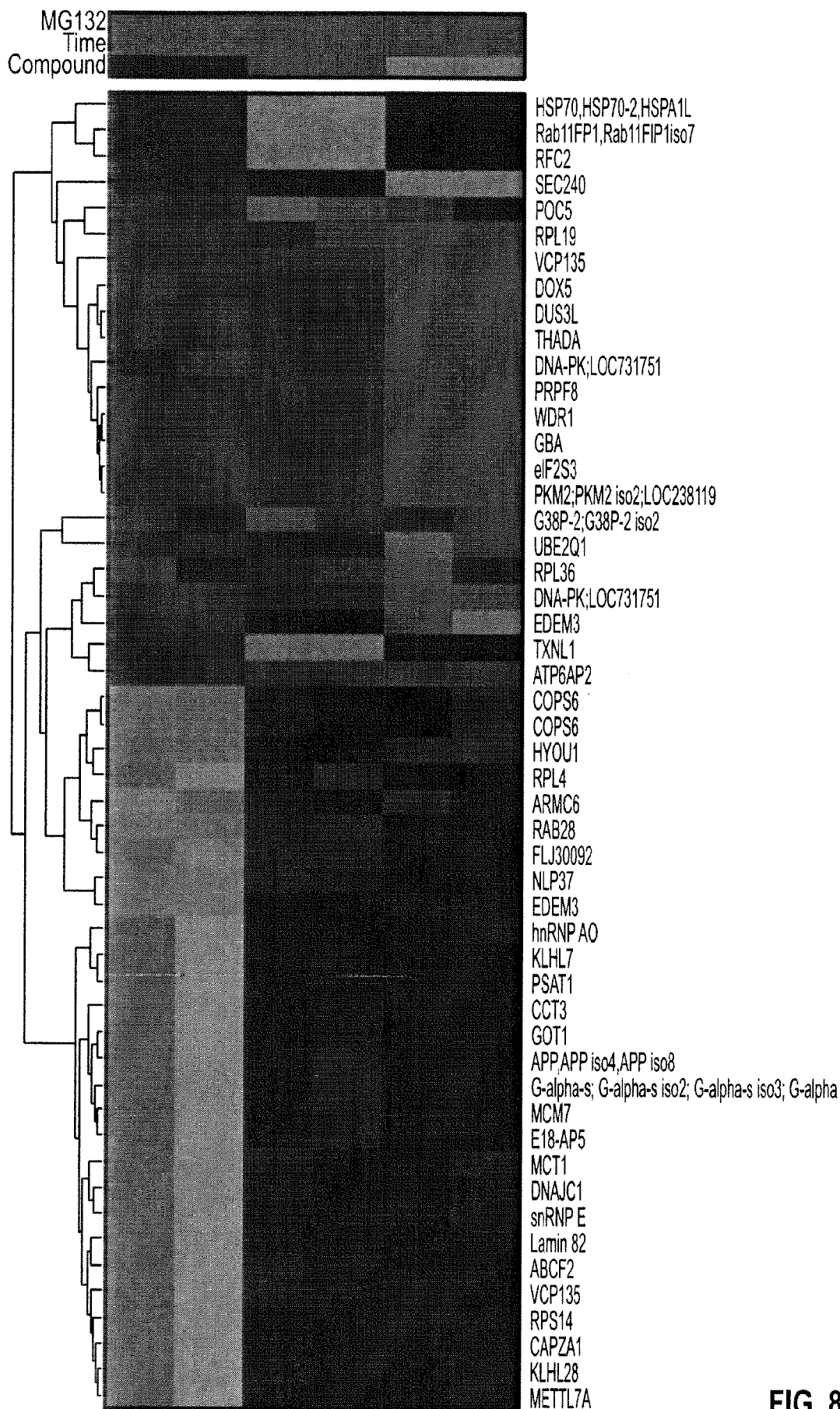


FIG. 8

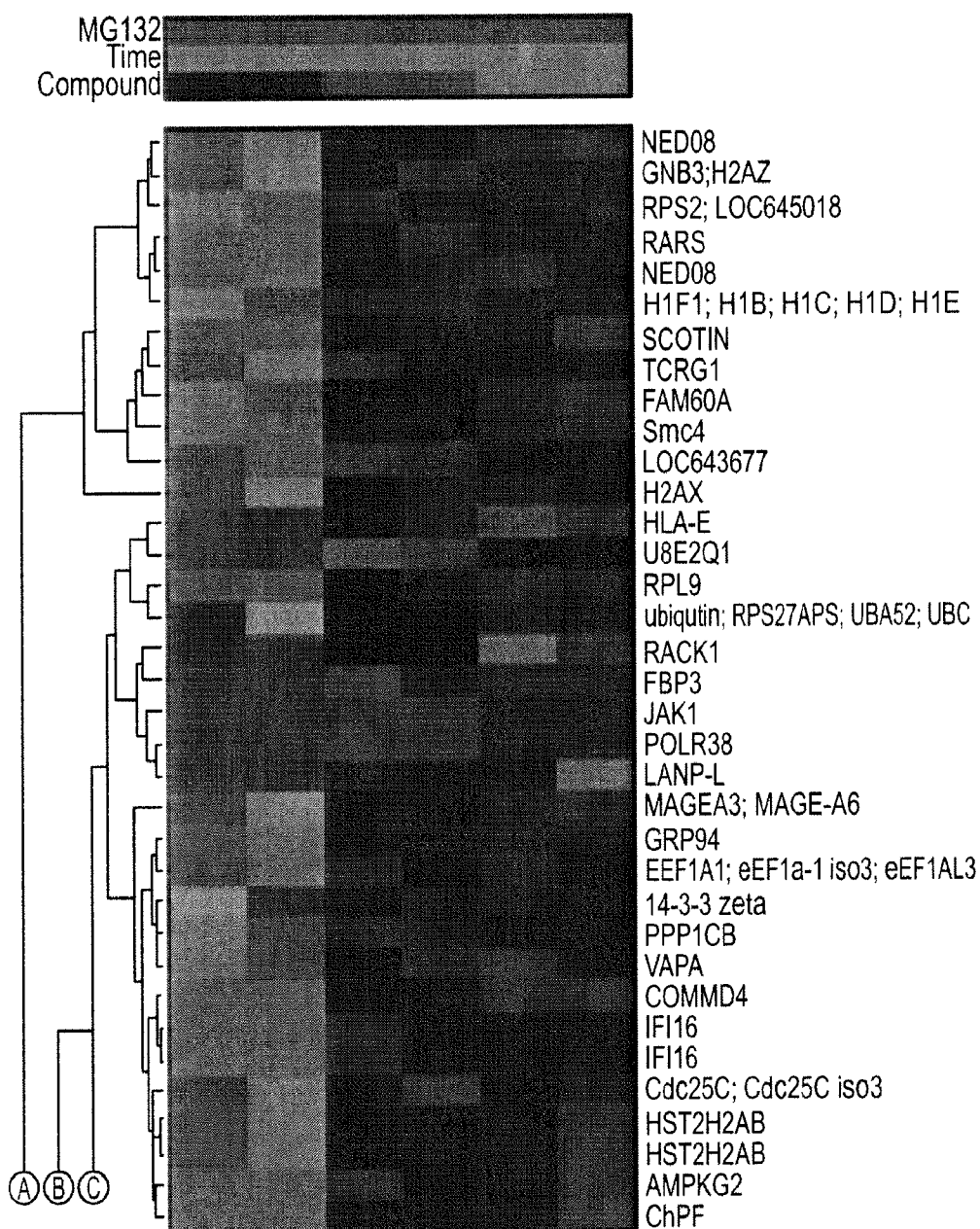


FIG. 9A

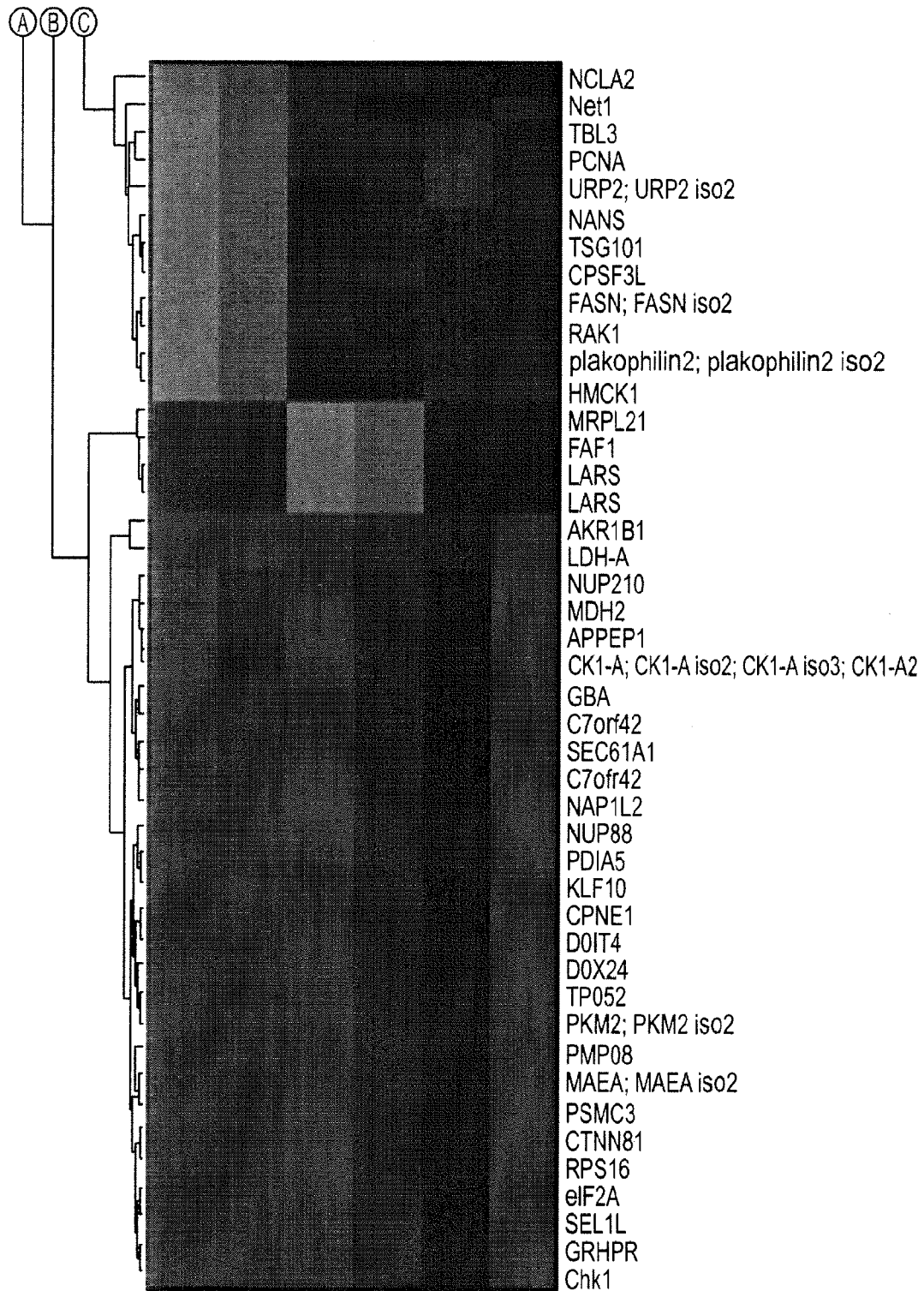


FIG. 9B

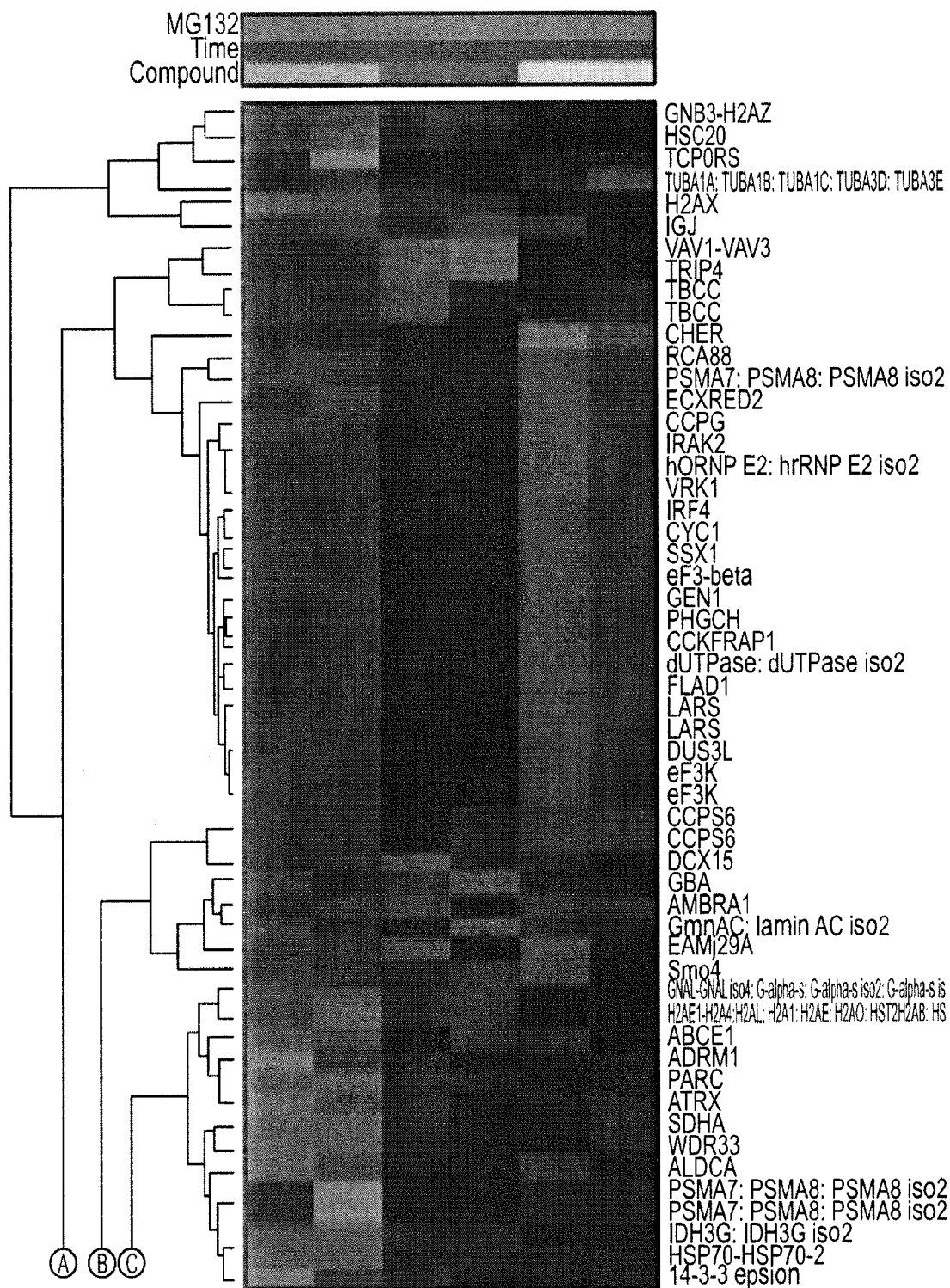


FIG. 10A

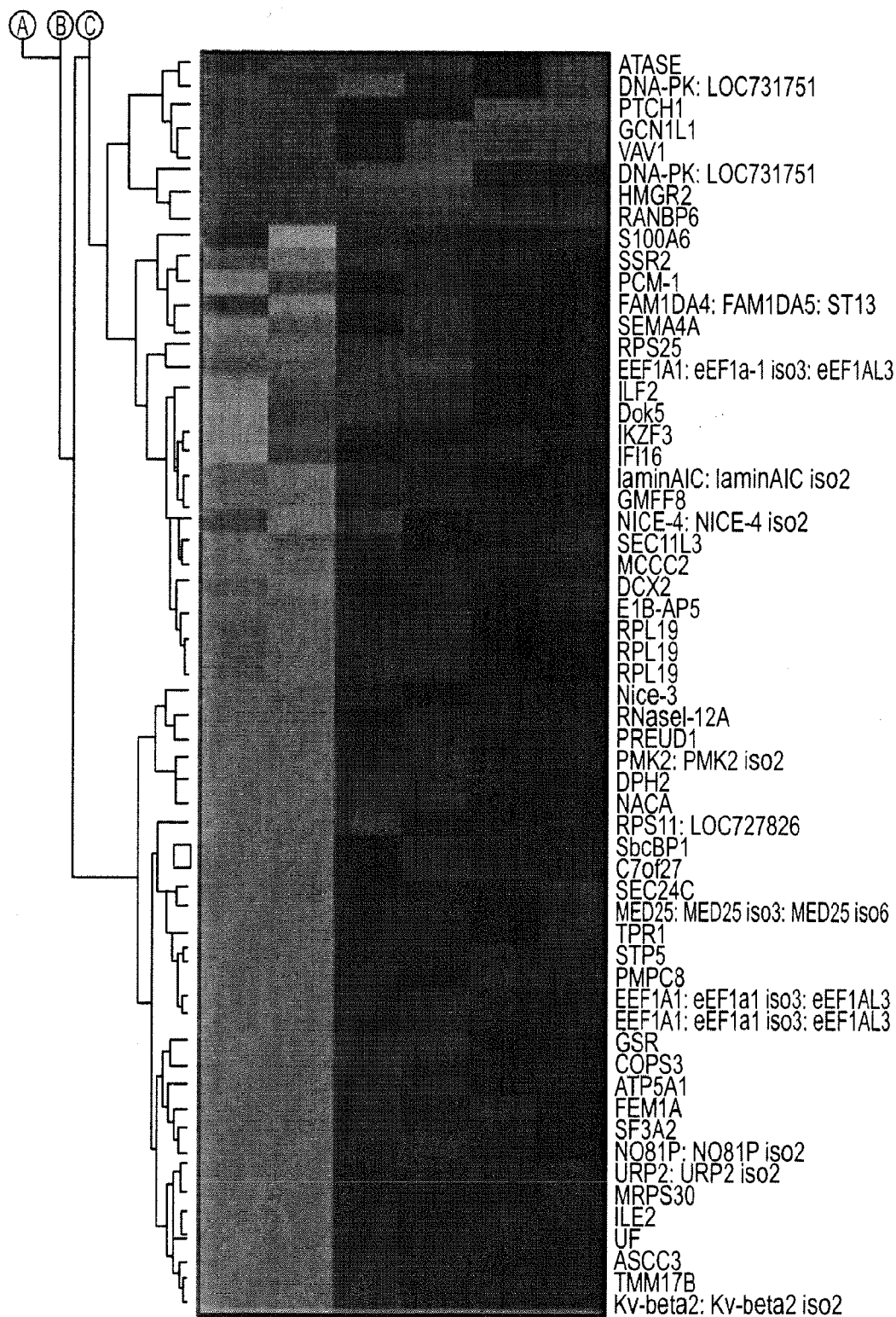


FIG. 10B

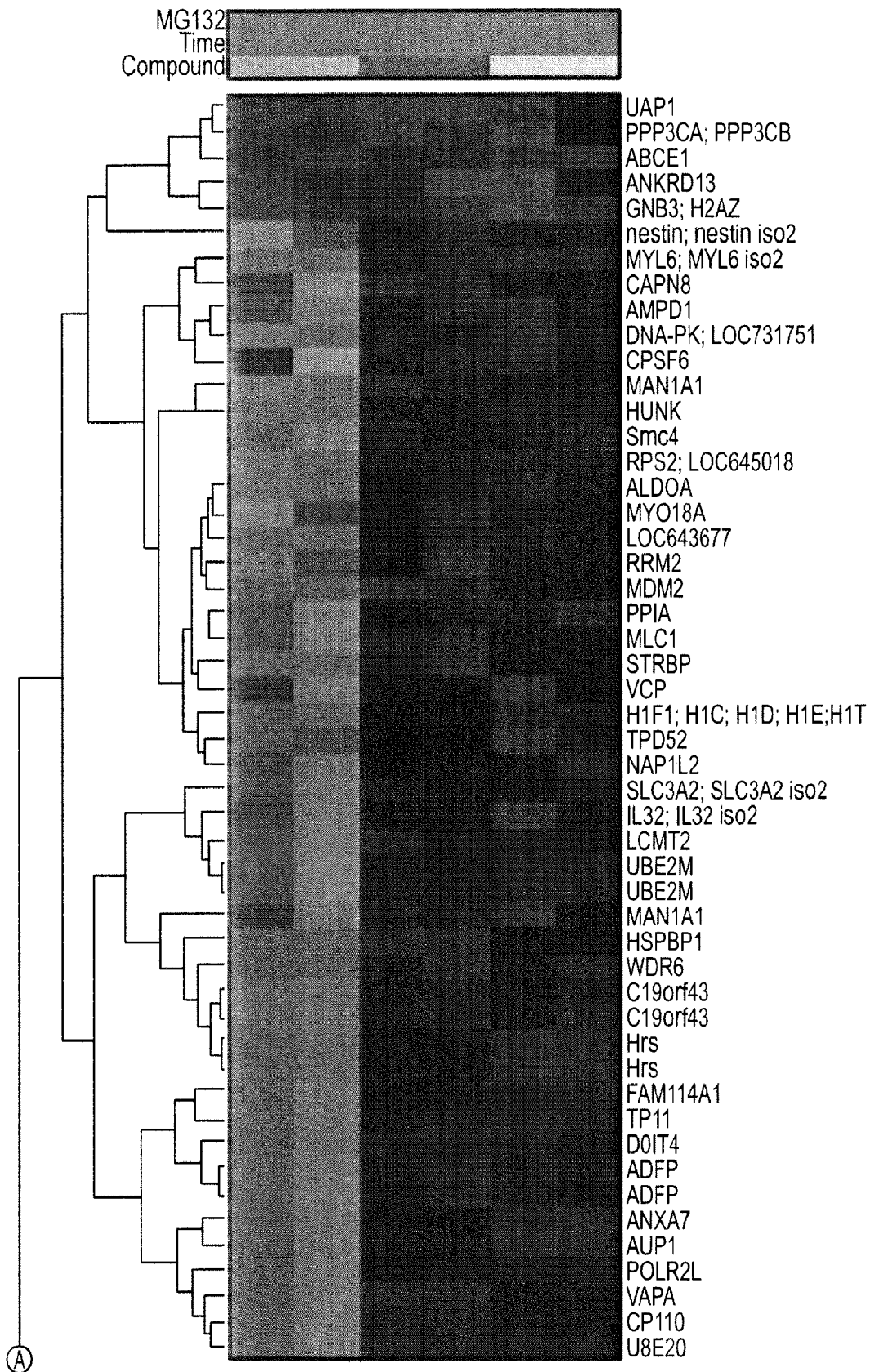


FIG. 11A

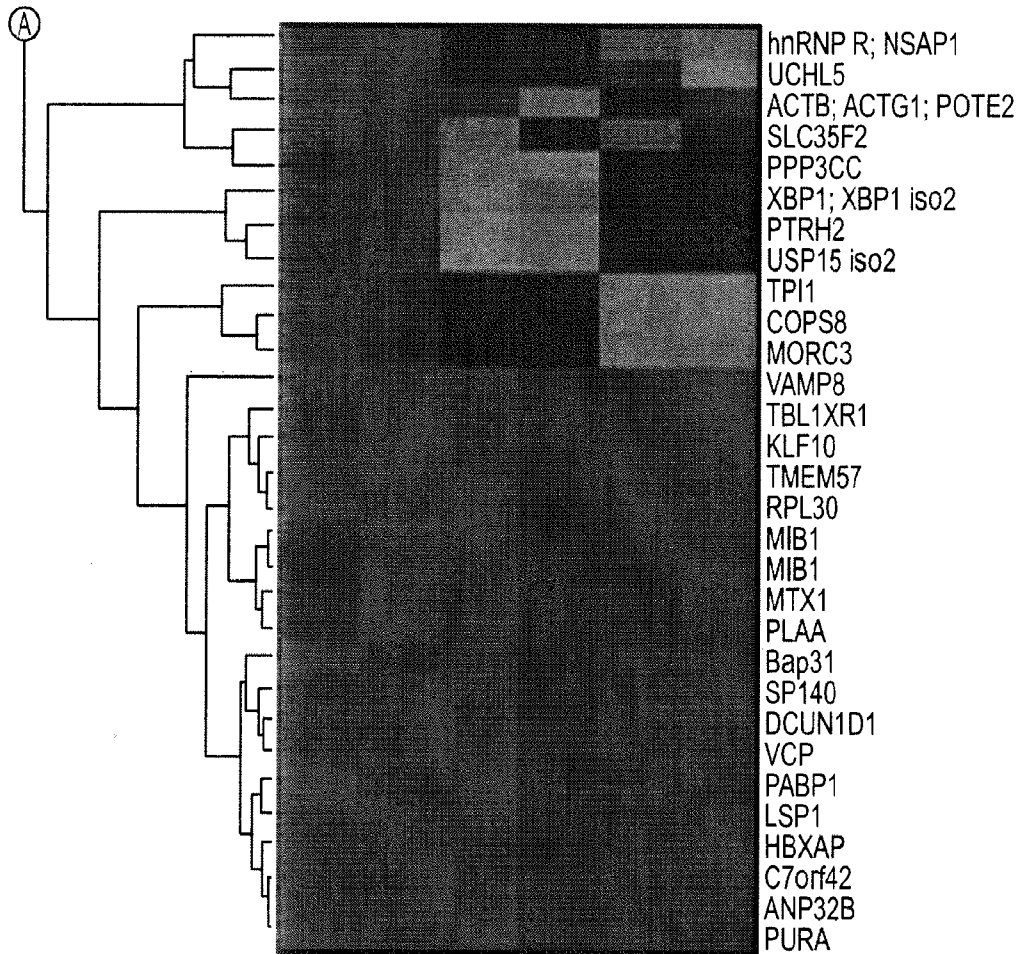
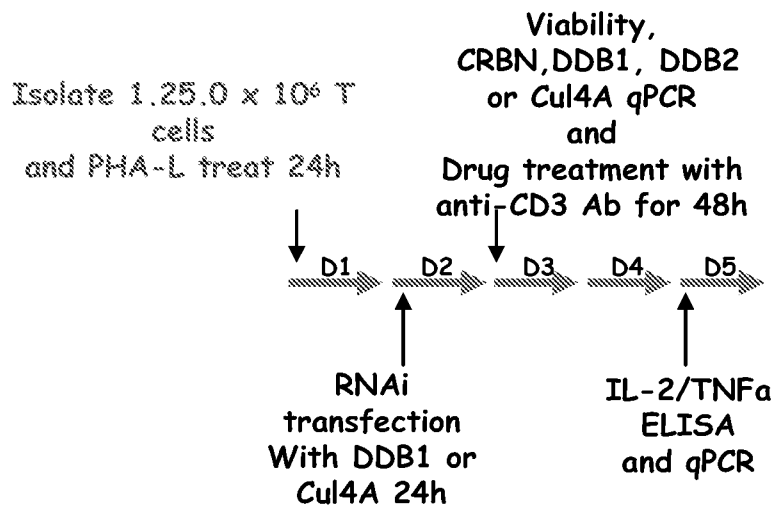


FIG. 11B

FIG 12A & 12B

A



B

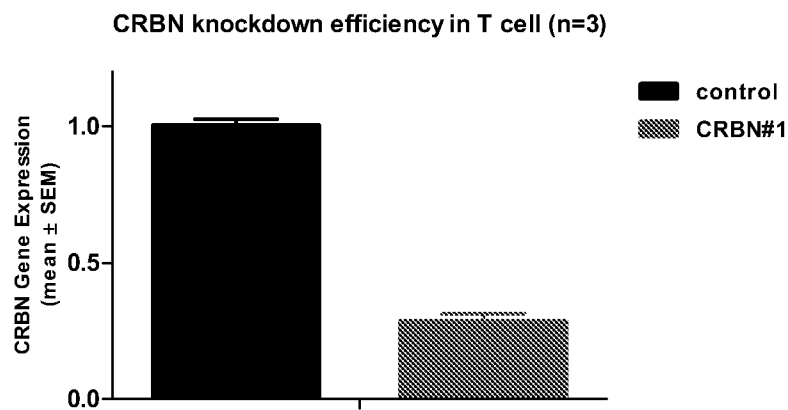


FIG 12C & 12D

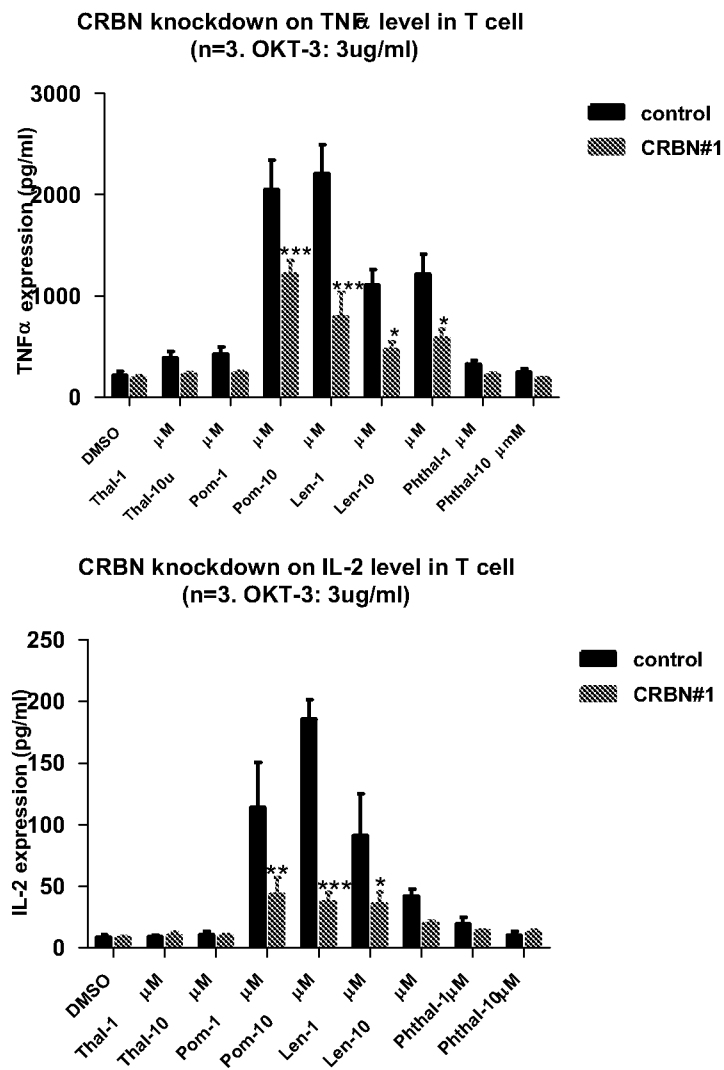
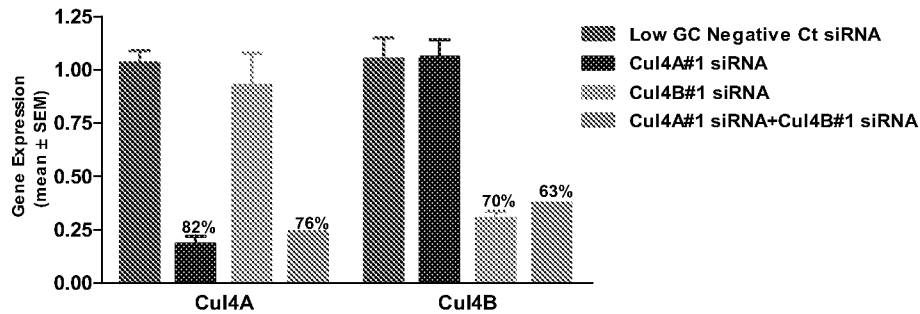


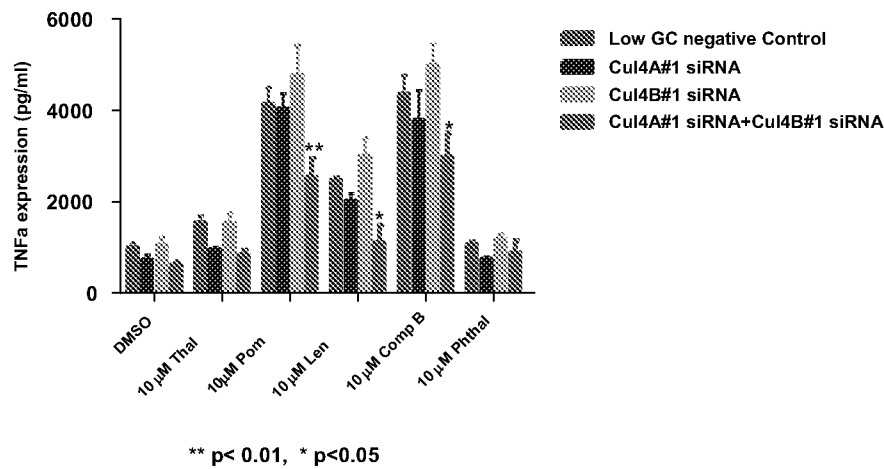
FIG 13A - 13C

Cul4A, Cul4B single or Cul4A+Cul4B double knockdown efficiency in primary human T cells (n=3)



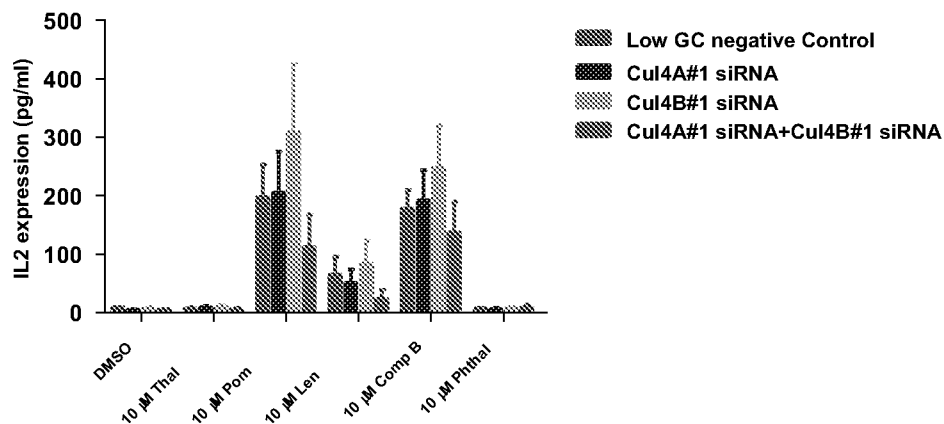
A

Cul4A, Cul4B single or Cul4A+Cul4B double knockdown on TNF α level in primary T cells (n=3. OKT3: 1 μ g/ml)



B

Cul4A, Cul4B single or Cul4A+Cul4B double knockdown on IL-2 level in primary T cells (n=3. OKT3: 1 μ g/ml)



C

FIG 14

Antiproliferative activity of CC-5013
vs baseline cereblon expression in DLBCL cells

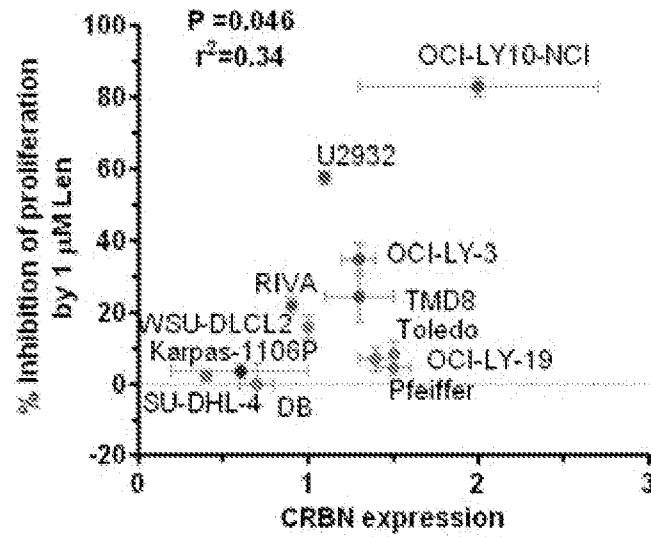
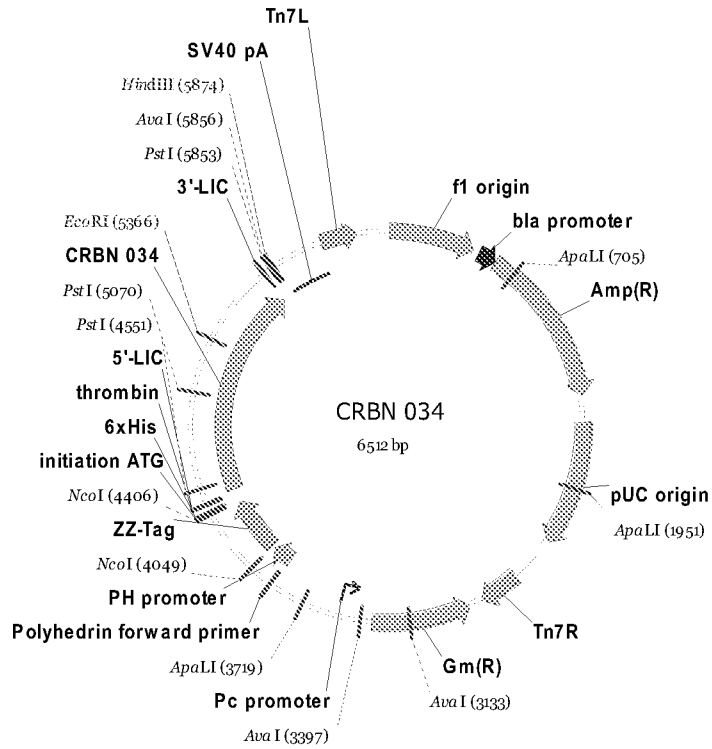
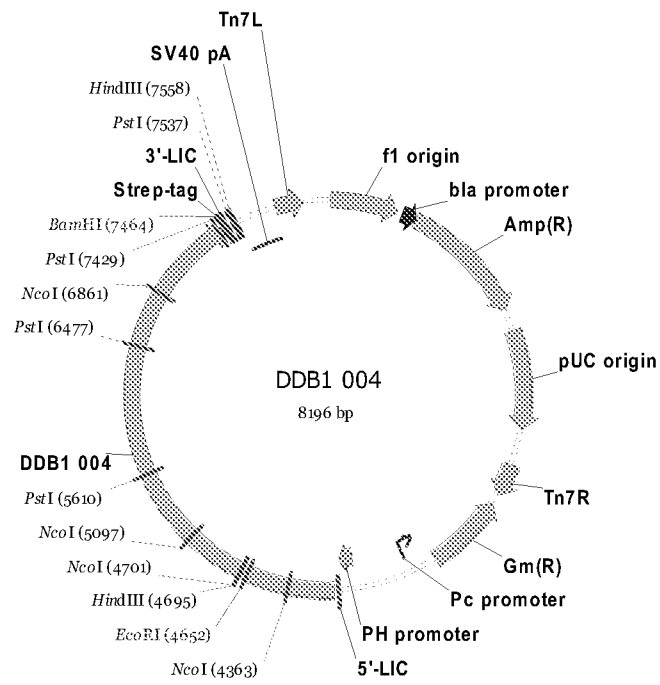


FIG 15A & 15 B

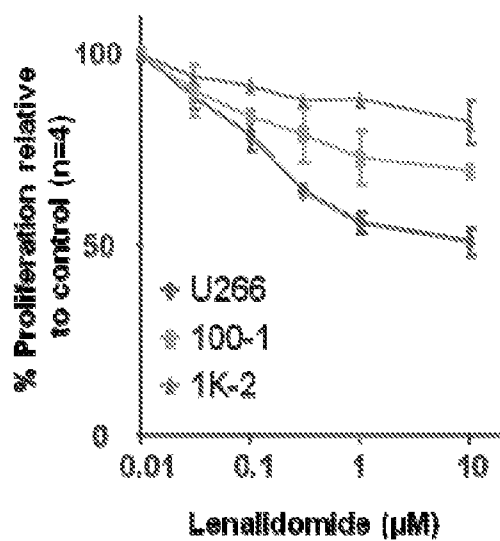


A

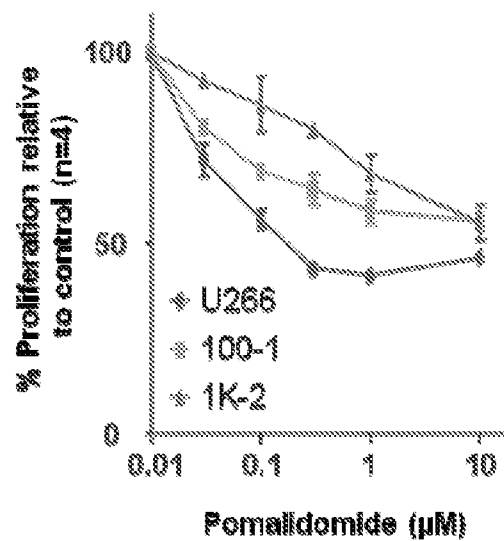


B

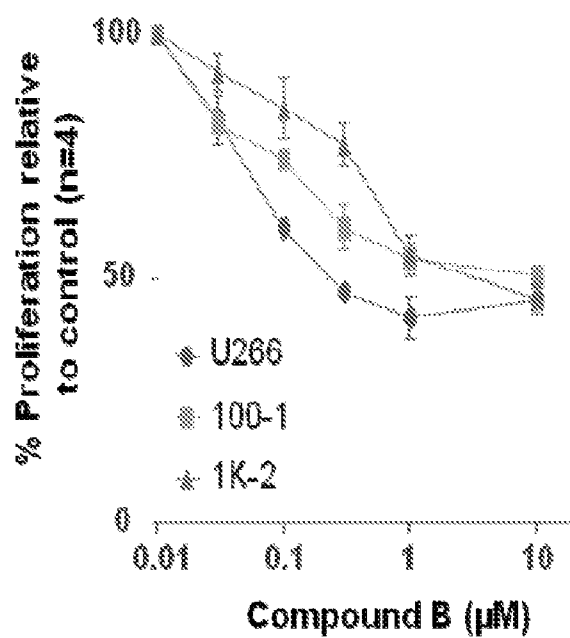
FIG 16A - 16 C



A



B



C

FIG 17

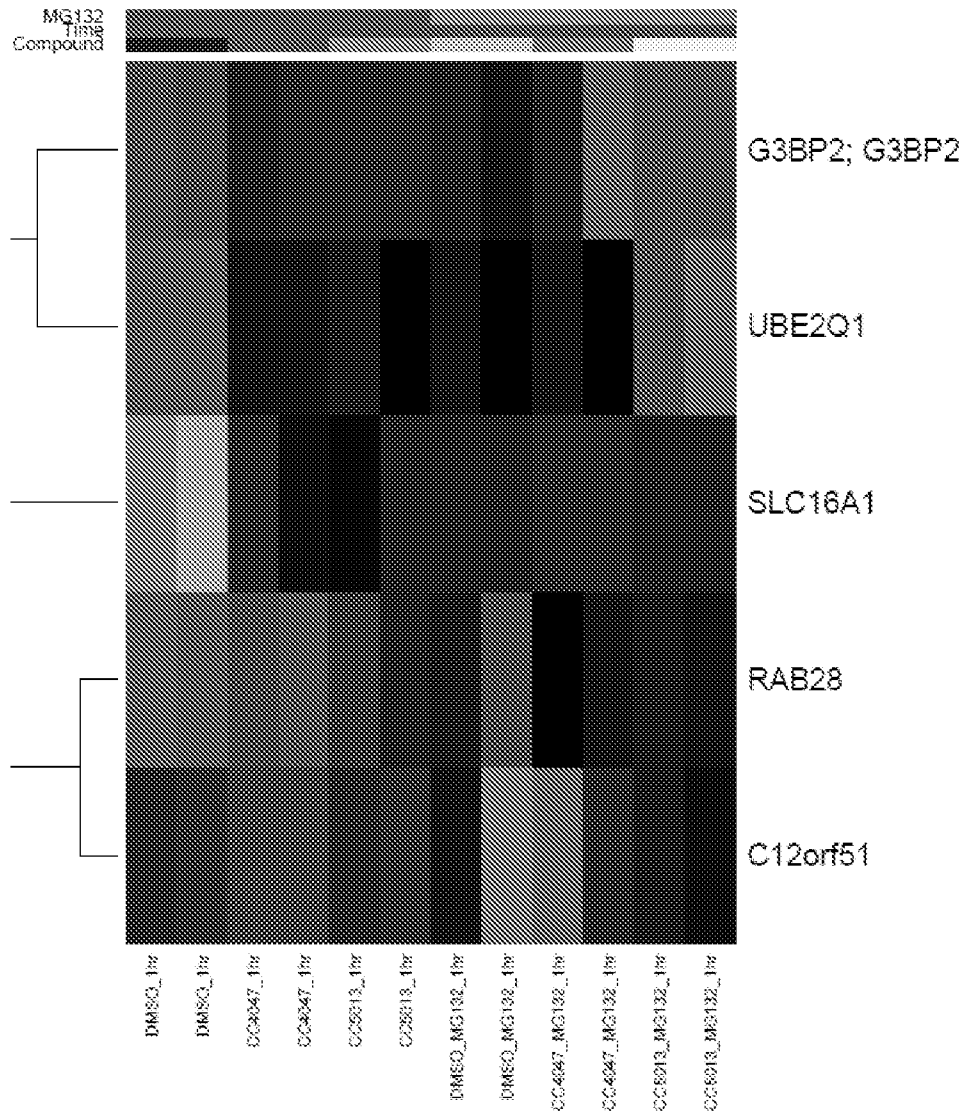


FIG 18

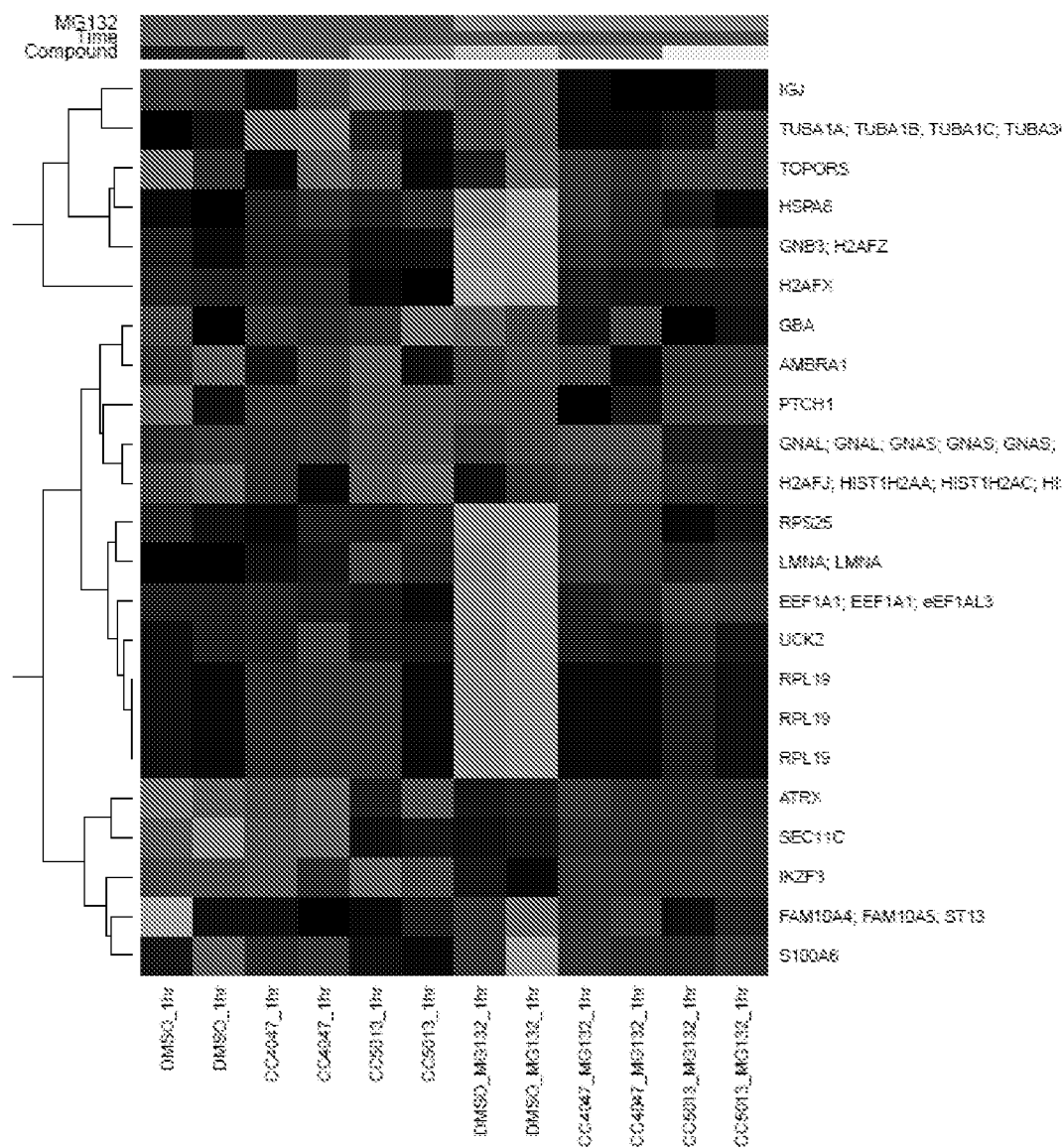


FIG 19

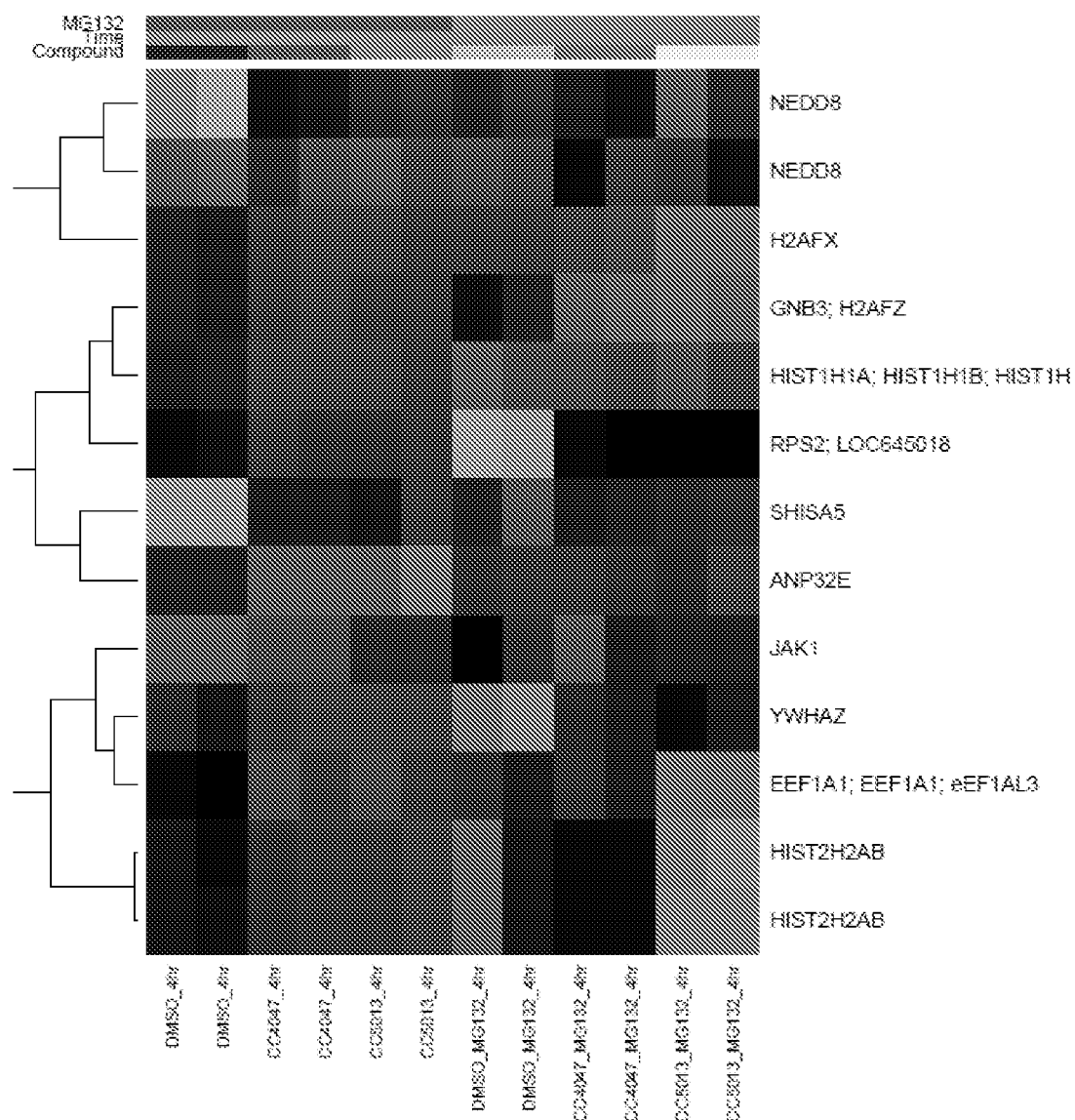


FIG 20

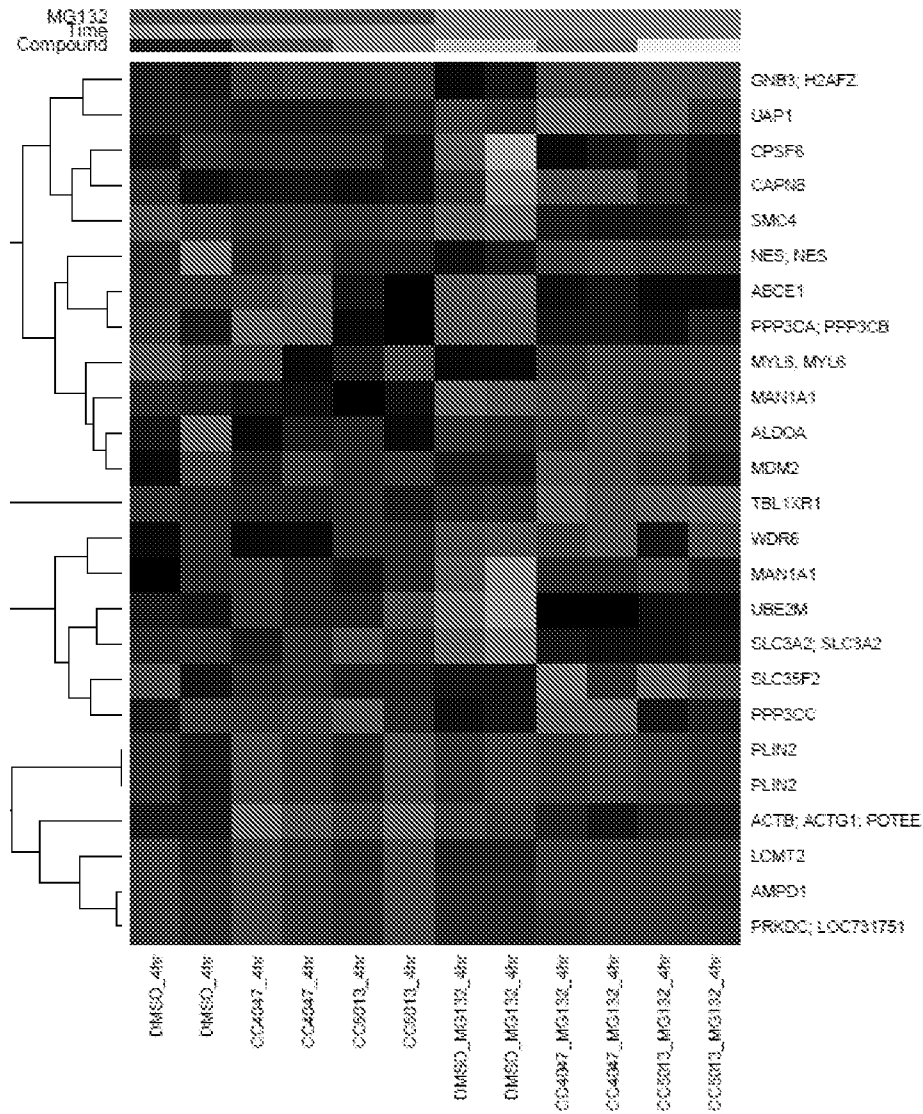


FIG 21

Gene Name	Peptide	Site	Fold Change	
			Pom 1hr	Len 1hr
UBE2Q1	ELK*LLESIFHR	46	-4.53	-7.59
G3BP2	QYYTLLNK*APEYLLHR	25	-2.19	-2.07
GNB2L1	LK*TNHITGYLNTVTVSPDGSLCASGGK	185	Pom 4hr	Len 4hr
			-2.08	-3.98

FIG 22

GeneName	Peptide	Site	Pom 1hr Fold Change	Len 1 hr Fold Change	Pom 4 hr Fold Change	Len.4 hr Fold Change	Pom/MG. 1hr Fold Change	Len/MG. 1h Fold Change	Pom/MG. 4h Fold Change	Len/MG. 4hr
C7orf42	QSNPEFCPEK*VA.	308			-161	-130			-94	-107
DUS3L	K*GGGCALM#NR	404	-35.5	-75.7			-37.9	-132		
GBA	VGLVASQK*NDLDA..	480	-53.1	-113	-201	-163				
KLF10	..VDEK*ASAALY..	223			-192	-155			-145	-165
LARS	VSELNAGIHK*TDQ..	807			-140	-2.43	-37.5	-130		
UBE2Q1	ELK*LLESIFHR	46	-4.53	-7.59	-3.23	-2.2				
ALDOA	..RPFPQVIK*SK	99					4.09	4.85		
ALDOA	VDK*GVVPLAGT..	111							4.73	4.54
C13orf40	KPDLRIEQUEEK*	6269			4.98	2.17			4.84	4.74
EEF1A1		450								
EEF1A		414								
eEF1A13	AAGAGK*VTK	450			2.91	2.94	11.3	3.32		
FERMT3	ETTLSYVK*SQDEAP..	385			108	210	129	174		
H2AFX	K*TSATVGP	120			2.65	2.71	7.19	3.97		
HNRNPUL1	..PFSYGYGGTGK*K	309	129	79.3			2.28	2.13		
RPS2		58								
LOC645018	AEDK*EWMPVTK	45			4.82	4.16			19.4	21.8

FIG 23

U266 multiple myeloma cell Ubiscan data with Lenalidomide and Pomalidomide

GeneName	Peptide	Site	Fold Change1 CC4047.1hr.FC	Fold Change2 CC5013.1hr.FC
RPL19	HMGIGK*R	80	-18.3	-26.7
GNE2L1	LK*TNHIGHTGYLNTVTVPDGLCAGGK	185	CC4047.4hr.FC	CC5013.4hr.FC
NEDD8	QMNDEK*TAADYK	54	-2.08	-3.98
HISTH1A	GTIVQTK*GTGASGSFK	100; -100; 97; -98; -97	2.45	2.87
NEDD8	LIYSGK*QMNDK	48	2.61	2.24
			2.15	2.68
PCM1	LMAAK*QK	666	CC4047.MG.1hr.FC	CC5013.MG.1hr.FC
HSPA8	DISENK*R	257	2.67	2.56
RPL19	TLSEKEETKK	190	4.01	2.83
RPL19	TLSEKEETK*K	195	2.46	2.06
RPL19	TLSEKEETKK*	196	2.46	2.06
IKZF3	SHTVEKPYK*CEFCGR	147	4	4.12
HNRNPR	IK*ALLER	128; 125	CC4047.MG.4hr.FC	CC5013.MG.4hr.FC
MIB1	SSEDATDDISSGNIPVLQK*DKDNTNVNADVQK	936	-9.66	-35.8
MIB1	SSEDATDDISSGNIPVLQKDK*DNTNVNADVQK	938	-100	-114
			-100	-114

T Cell Ubiscan data with Compound B

Gene Name	Peptide	Site	CC122 p.val	CC122FC
a → IKZF3	EYNEYENIK*LER	100	0.00526	29.70
a → RPL19	KK*LLADQAEAR	153	0.03800	18.00
a → PCM1	QQNQHPKPGGK*ER	1096	0.02600	3.33
a → MIB1	GQSPDLDCPDNLCALAK*	791	0.03400	1.96
a → NEDD8	VK*TLTGKEIEDIEPTDKVER	6	0.04240	1.57
b → HSPA8	ILDKCNEHNWLDKNQTAEK*EEFEHQQKELEK	589	0.04270	-1.58
b → HSPA8	ILDKCNEHNWLDKNQTAEK*EEFEHQQK*ELEK	597	0.04270	-1.58
b → GNE2L1	LWNTILGVCK*YTVQDSEHSEWVSCVR	139	0.04430	-1.87
b → HISTH1A	SLVSK*GTIVQTK	93; 93; 90; 91; 90	0.03220	-1.92
b → RPL19	K*PVTVHSR	53	0.02390	-2.33
b → HNRNPR	IK*ALLER	128; 125	0.02520	-2.95

a: Ub-proteins that are commonly increased in abundance by Rev and Pom in U266 and Compound B in T cells

b: Proteins that are commonly decreased in abundance by Rev and Pom in U266 and Compound B in T cells

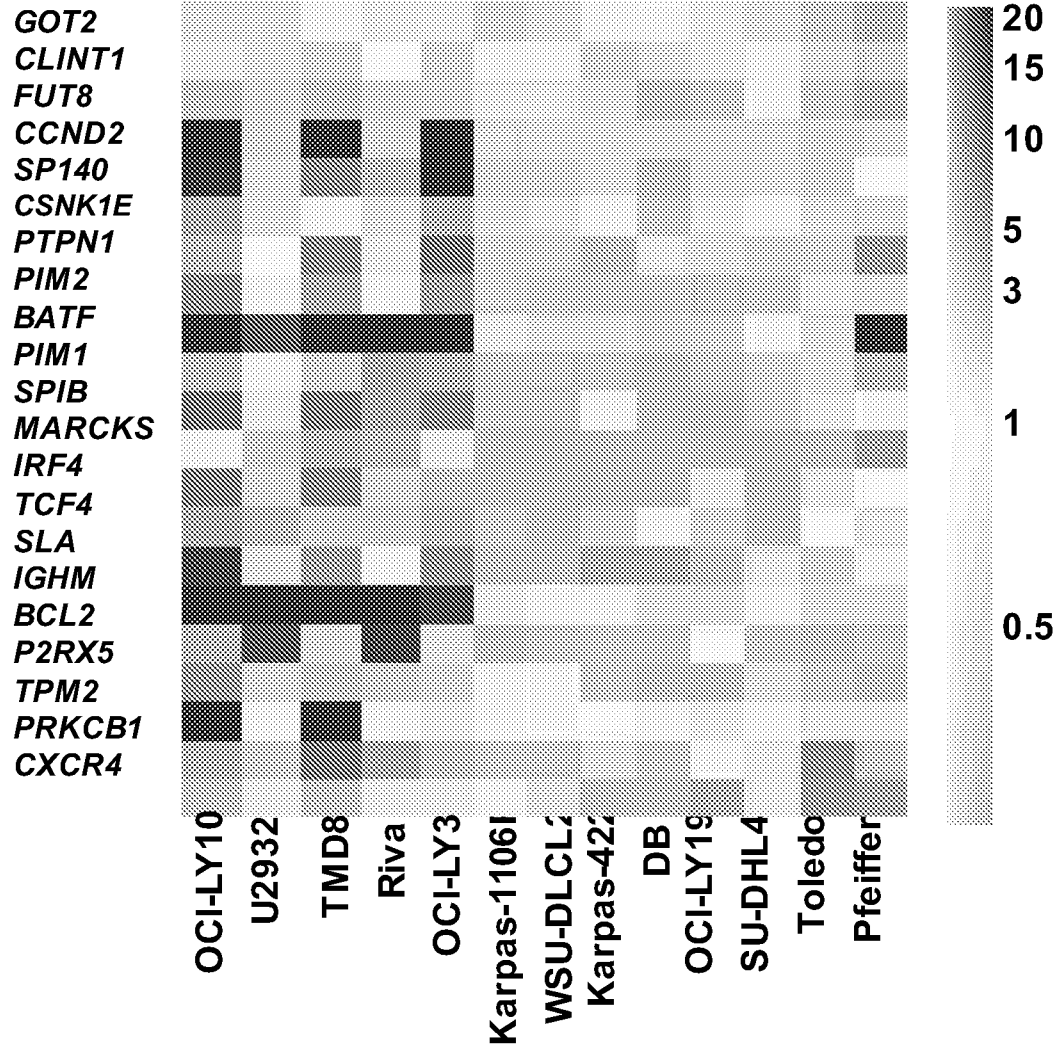
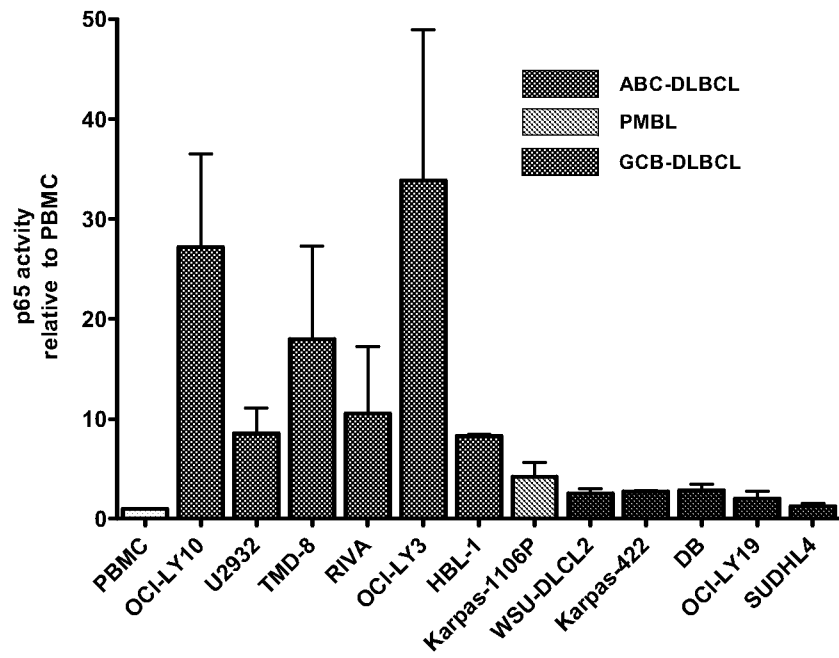


FIG. 24B

Baseline NF κ B p65 activity in DLBCL cells

Baseline IRF4 expression

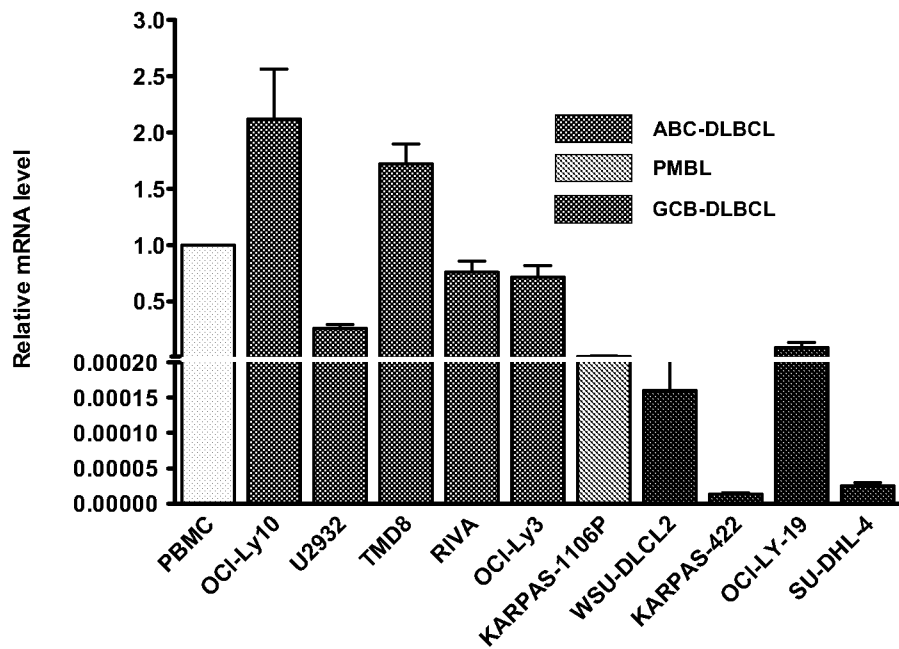
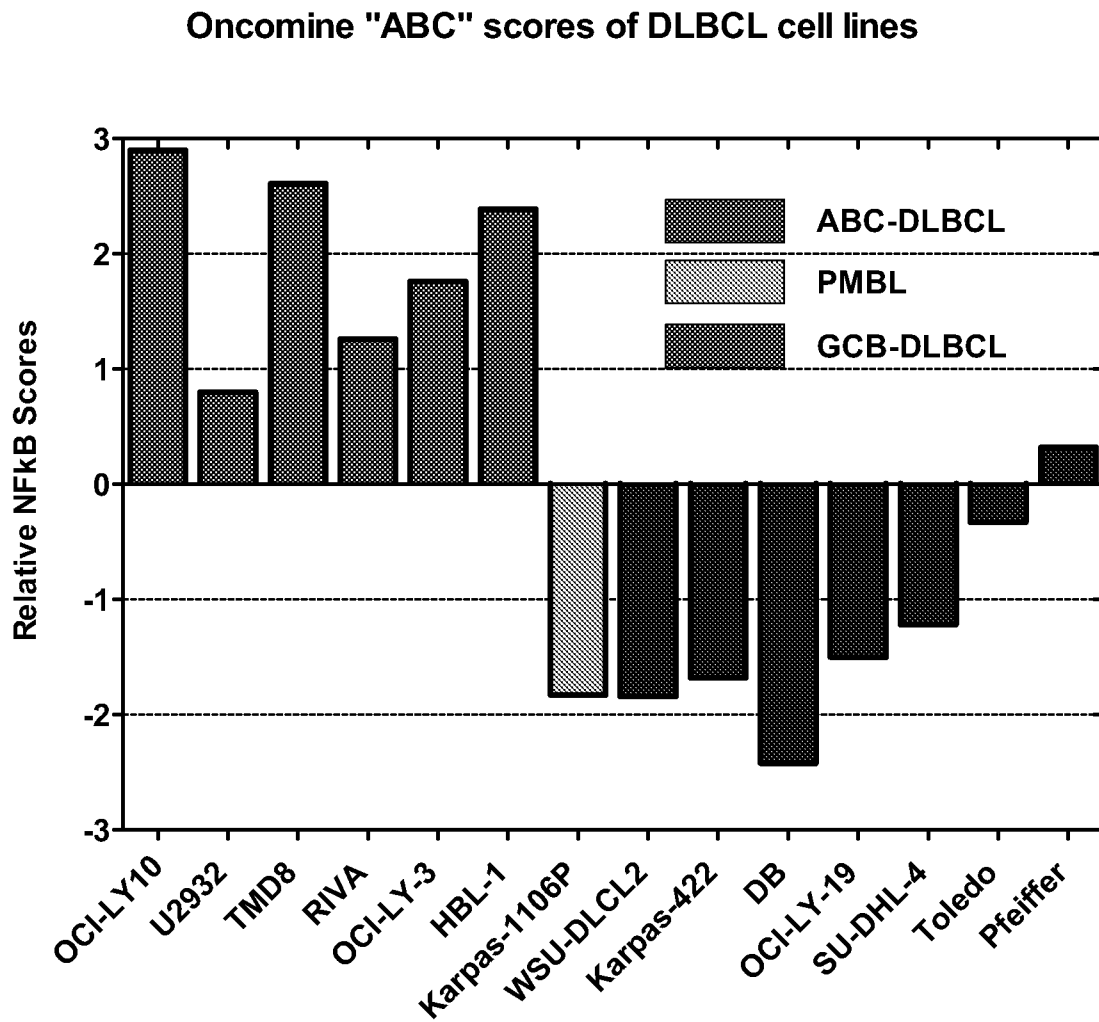


FIG. 24C



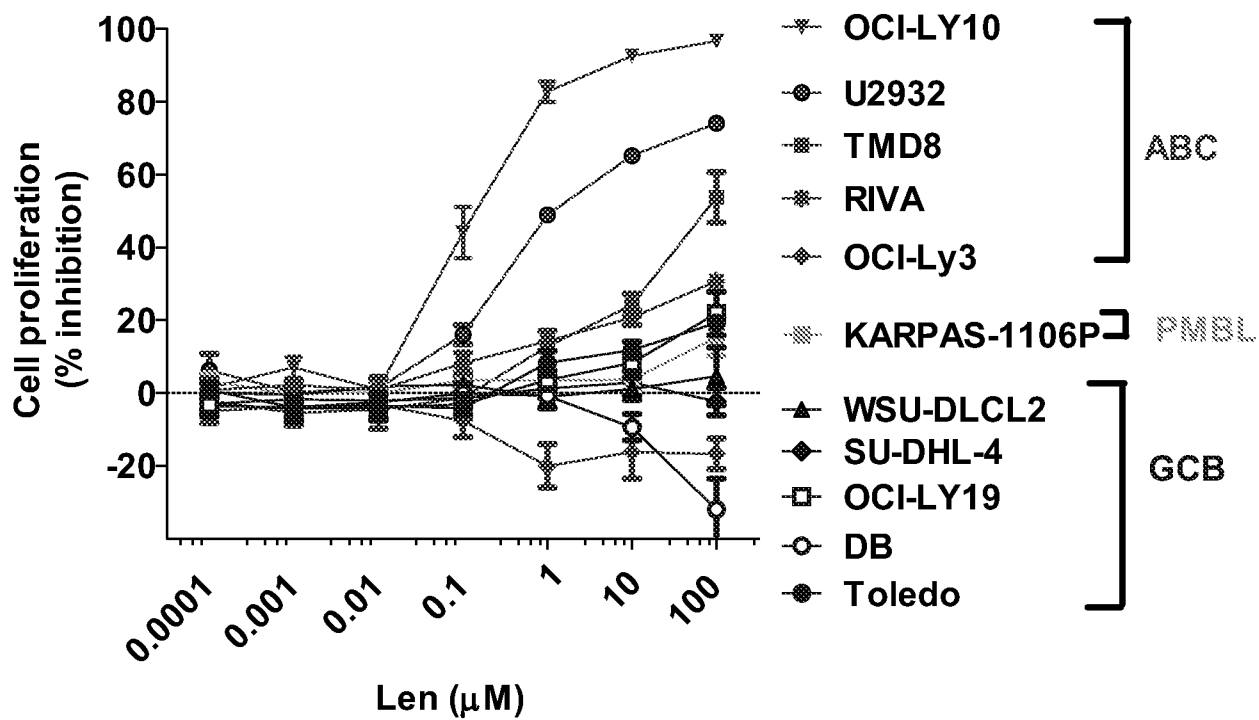


FIG 26

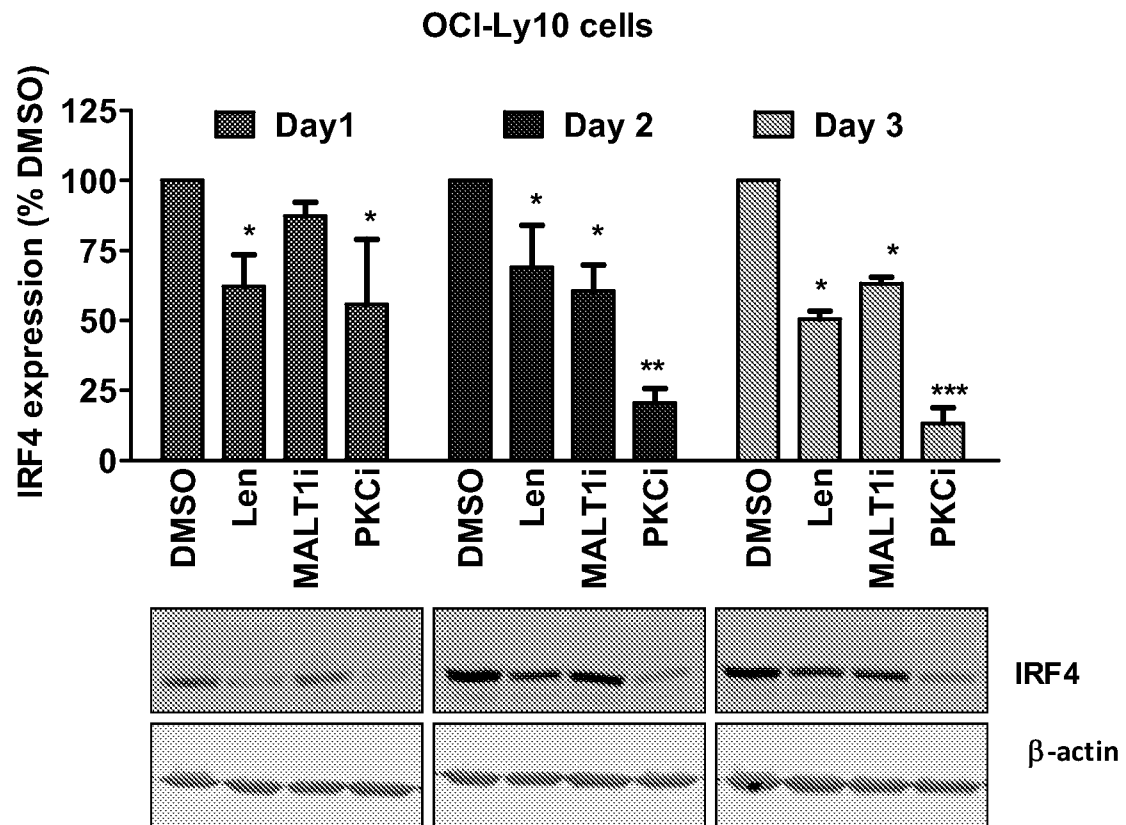


FIG 27A

U2932 cells

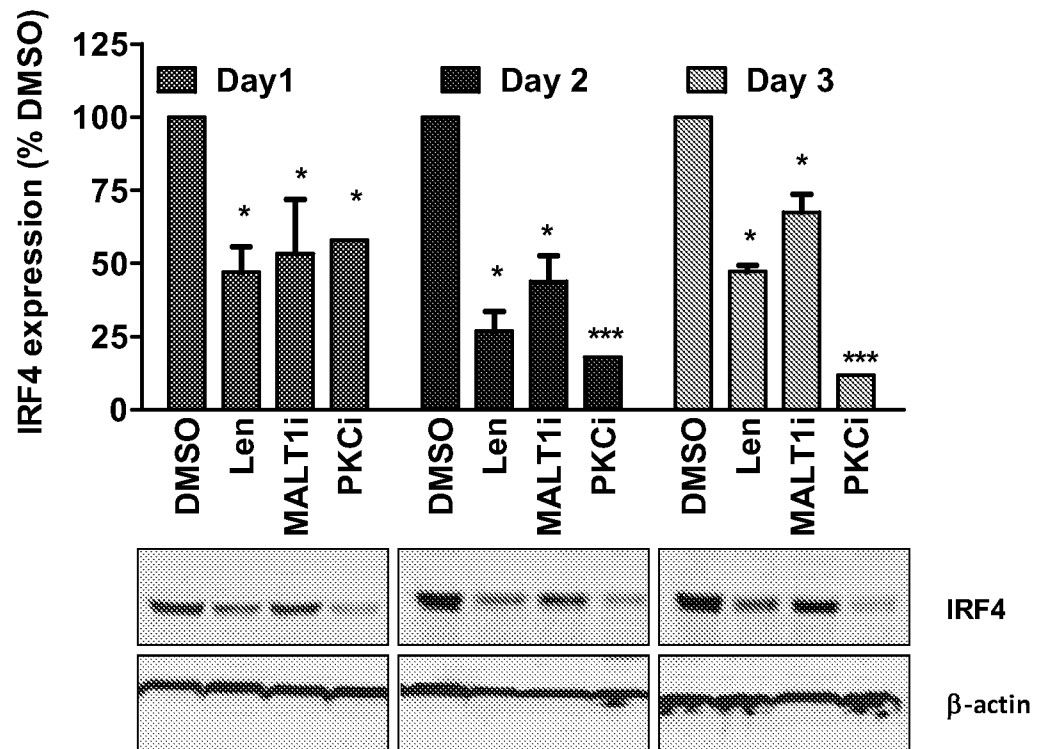


FIG 27B

OCI-Ly3 cells

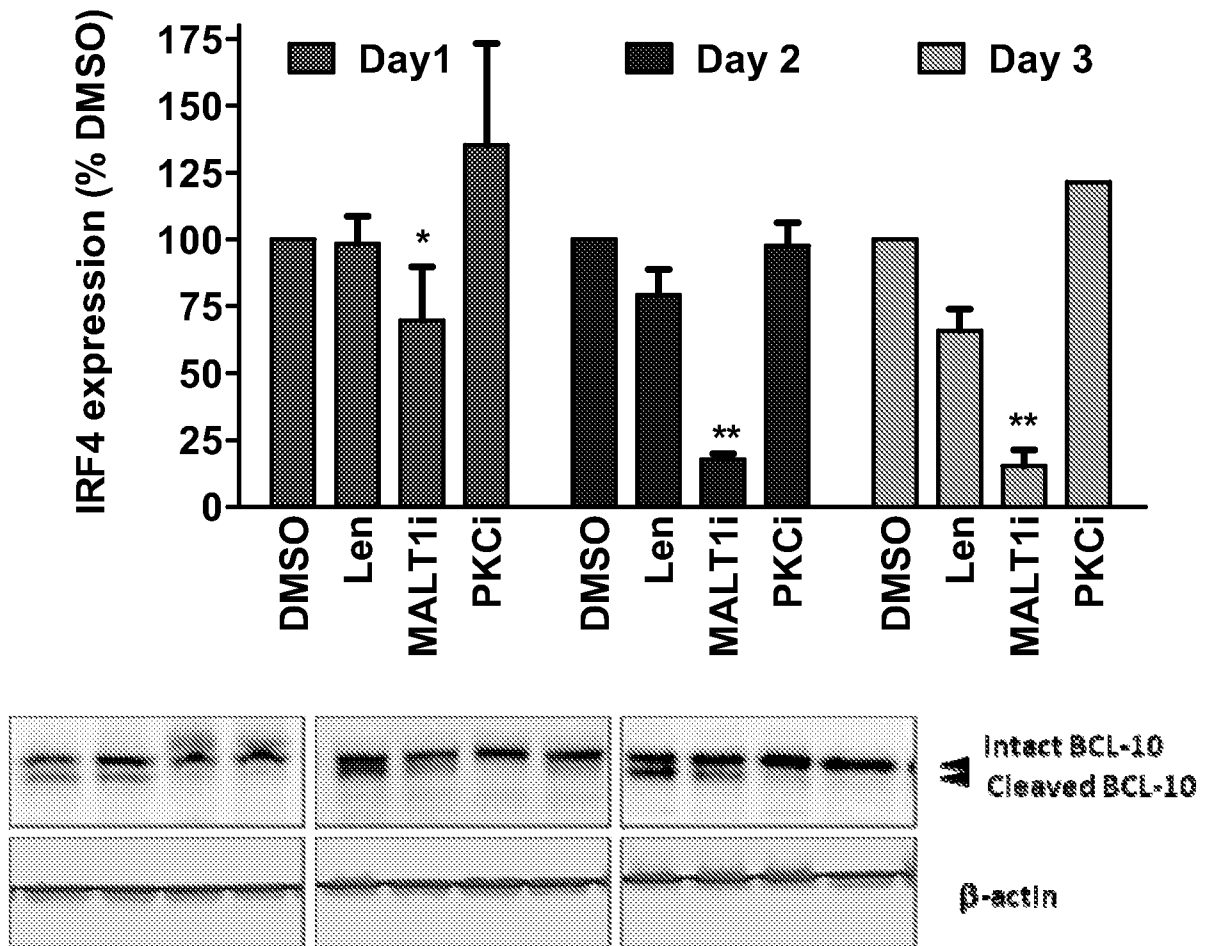


FIG 27C

BCL-10 cleavage in OCI-Ly10 cells (n=4)

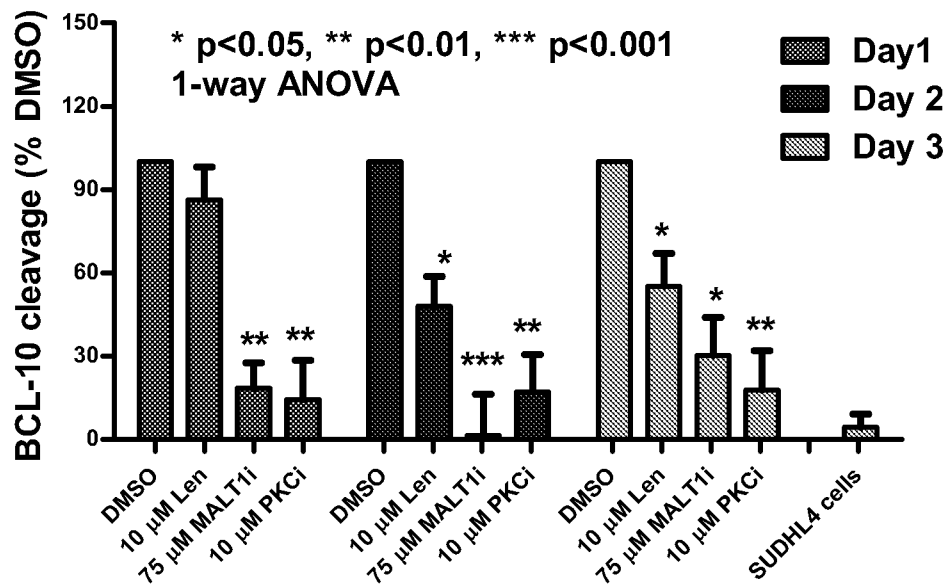


FIG 28

GGGCCGATTTTCAATGTCATTCTTCAGTTTTGAGACTGATTCTCTCCAGC OCI-Ly3
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC U2932
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC OCI-Ly10
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC RIVA
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC Karpas422
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC TOLEDO
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC TMD8
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC OCI-Ly19
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC Karpas1106p
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC SUDHL4
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC WSU-DLCL2
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC HBL1
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC Pfeiffer
GGGCCGATTTTCAATGTCATTCTTCAGTTTTAGAGACTGATTCTCTCCAGC DB

Mutation analysis of CARD11 coiled-coil domain 1 in DLBCL cells

FIG 29A

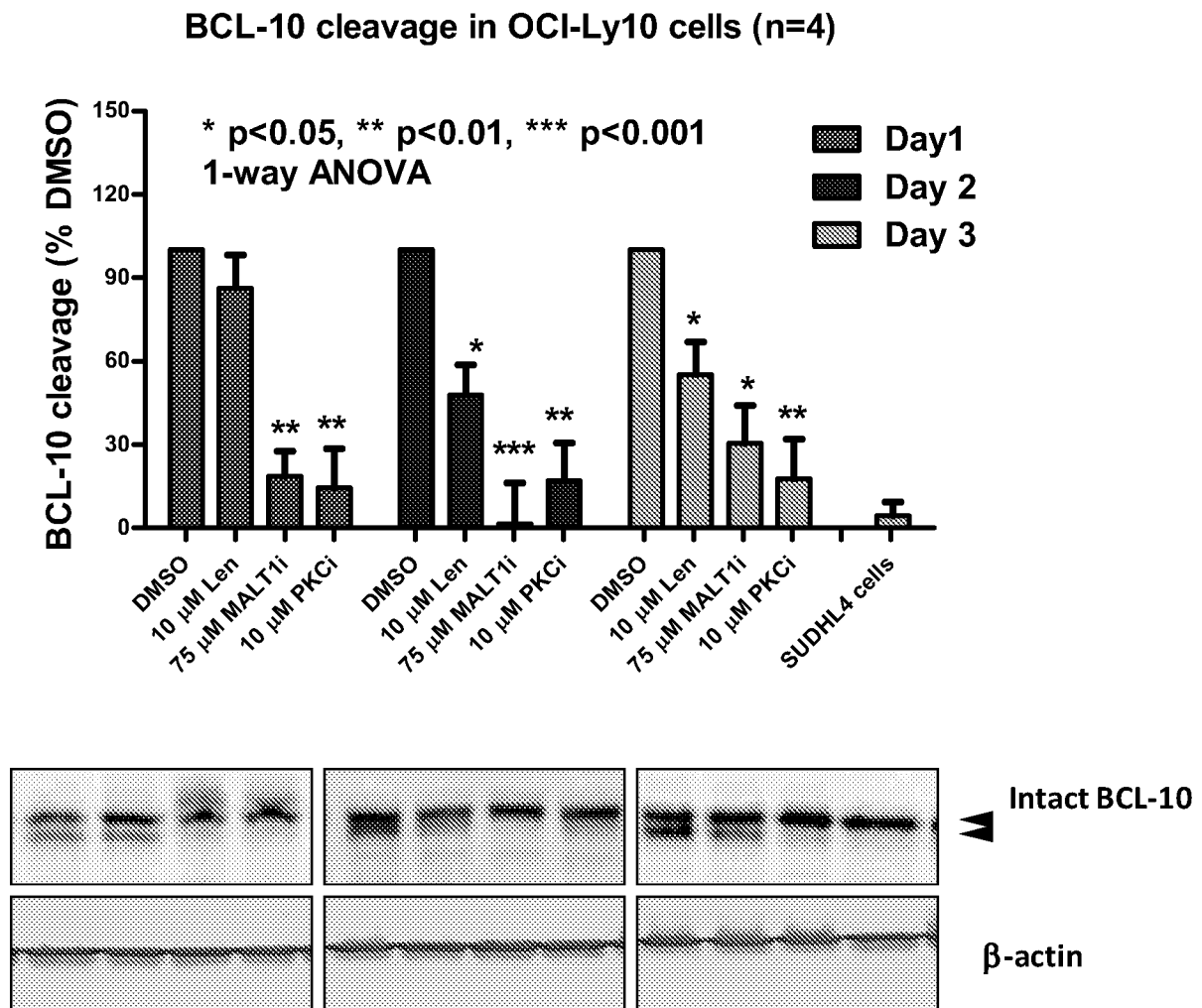


FIG 29B

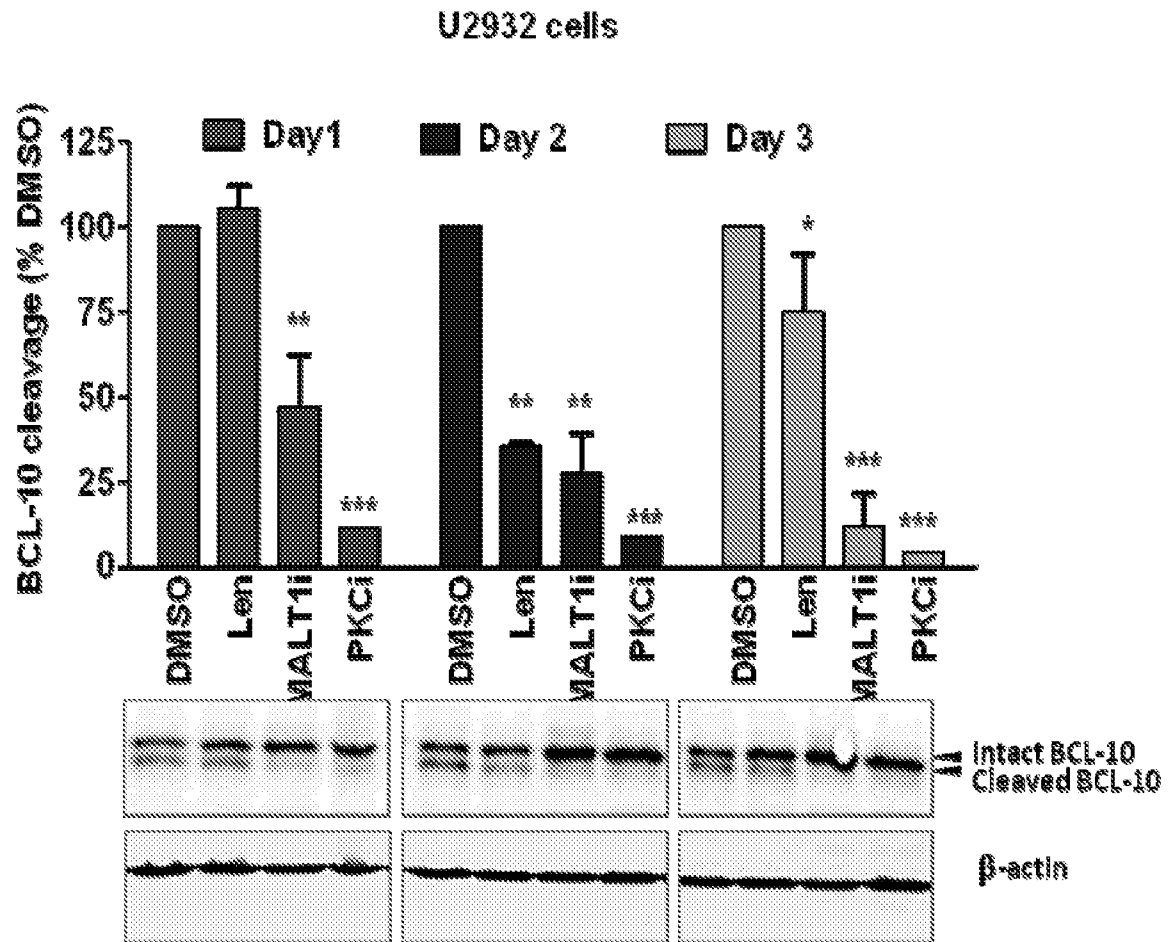


FIG 29C

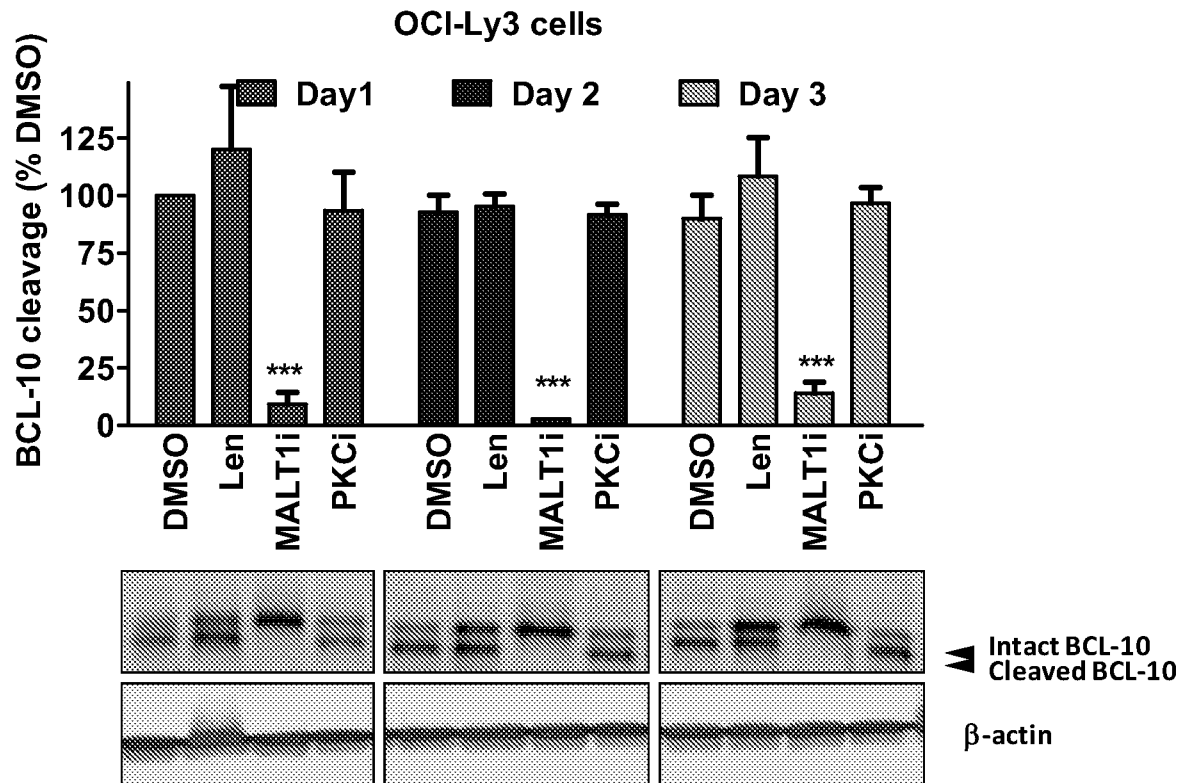


FIG 30A & 30B

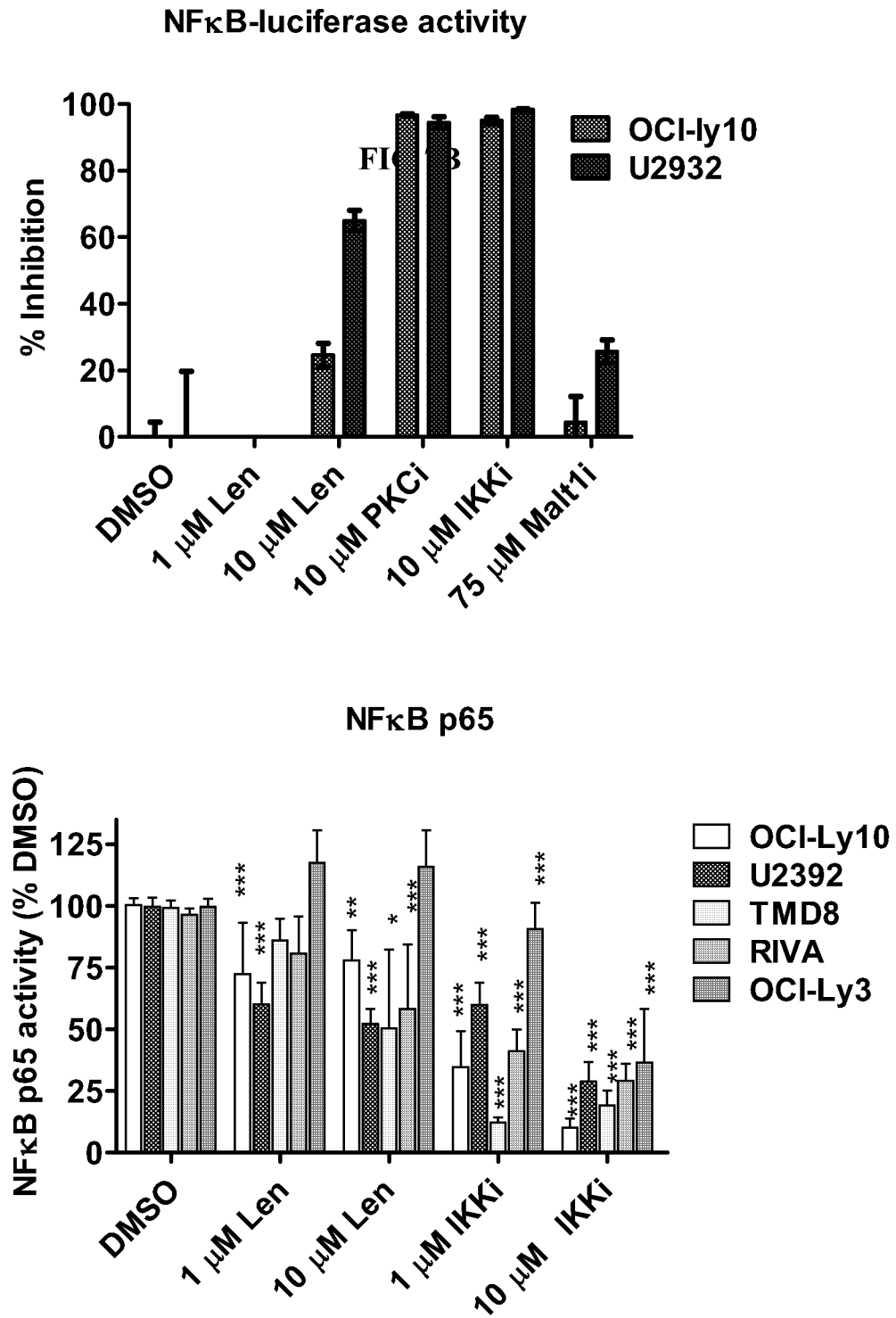


FIG 30C

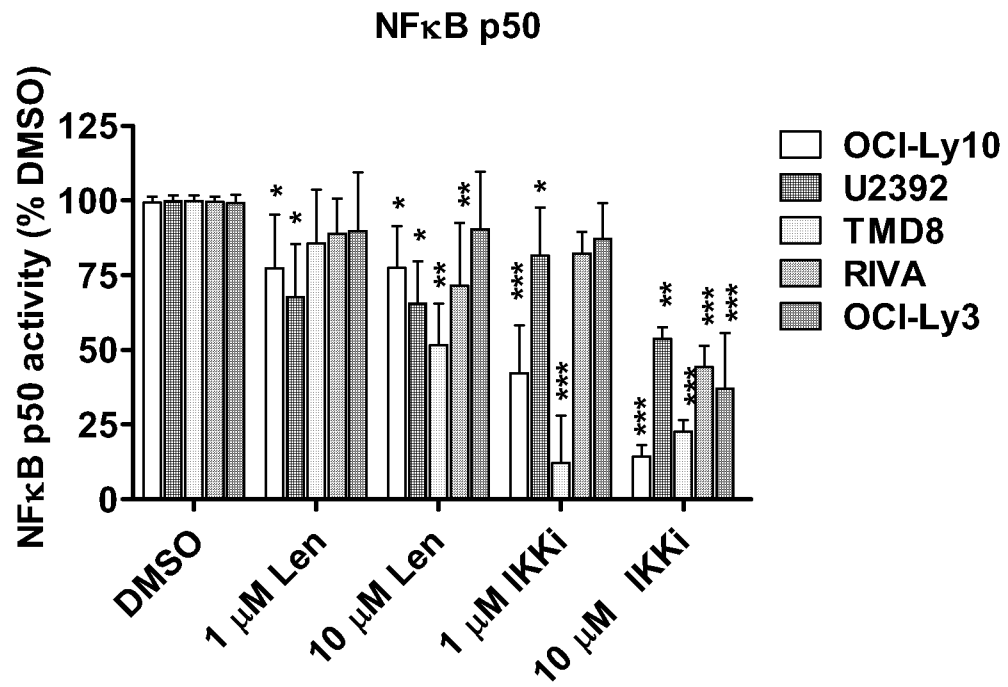


FIG 31A & 31B

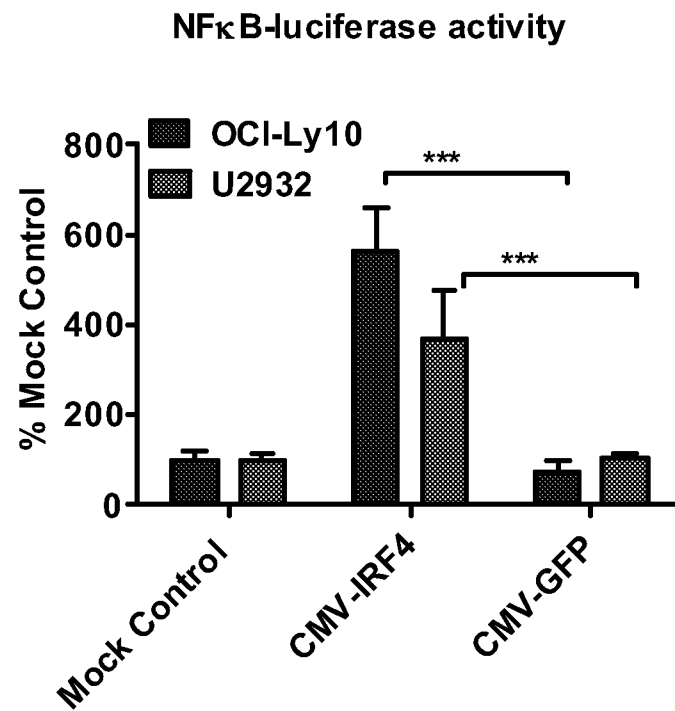
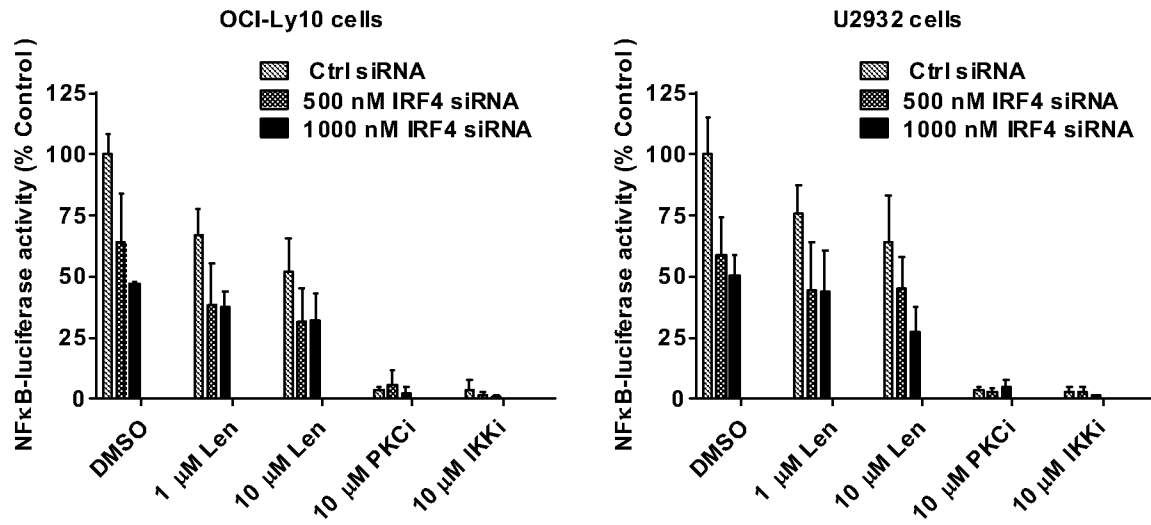


FIG 31C

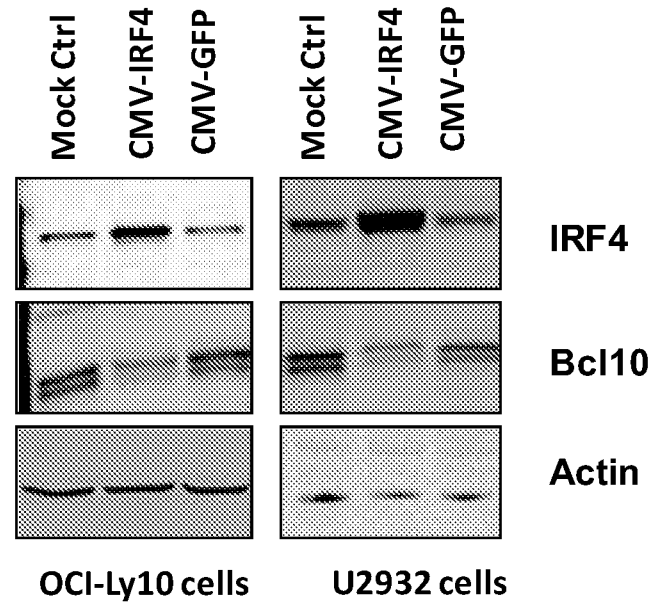


FIG 31D

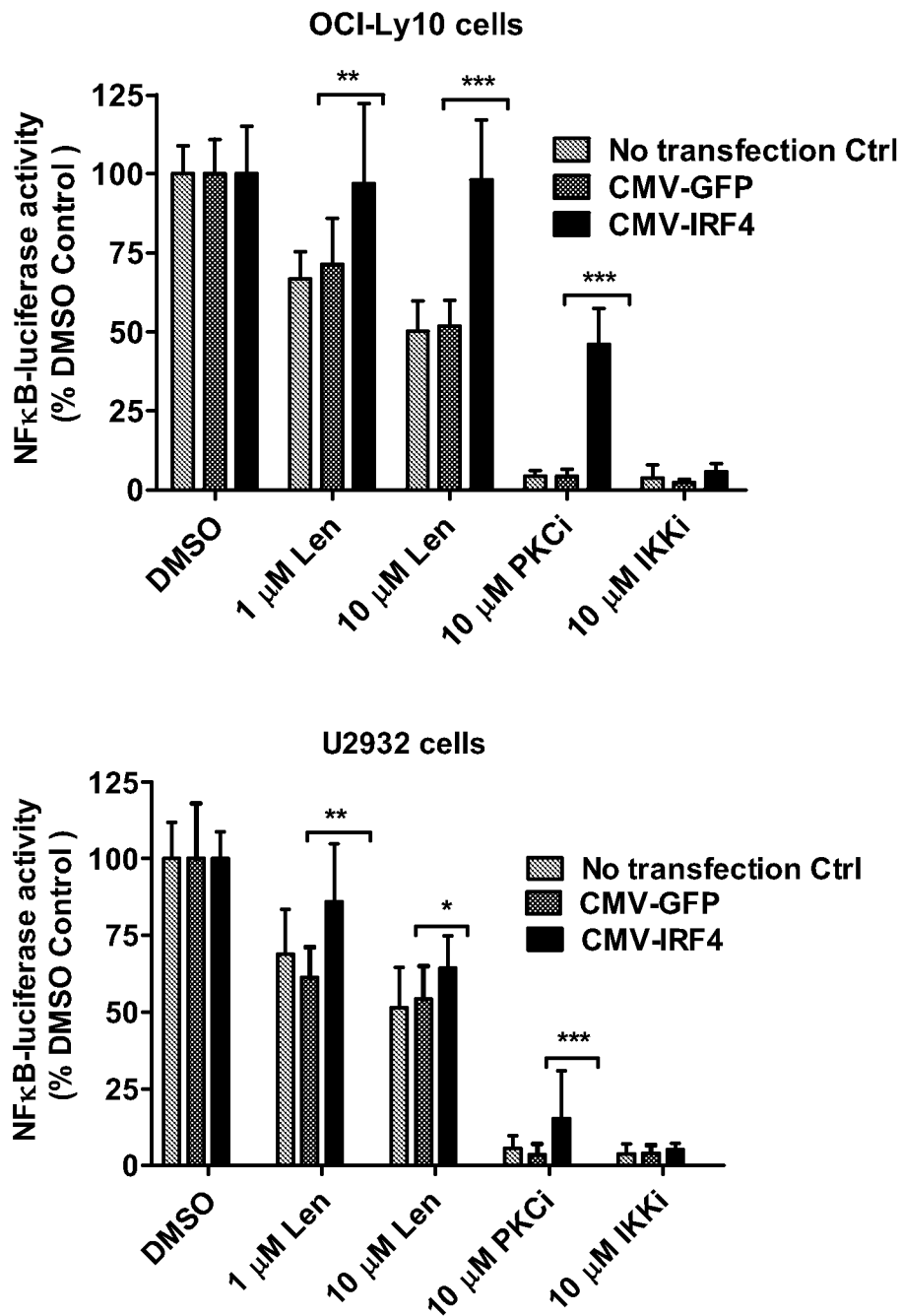


FIG 32A & 32B

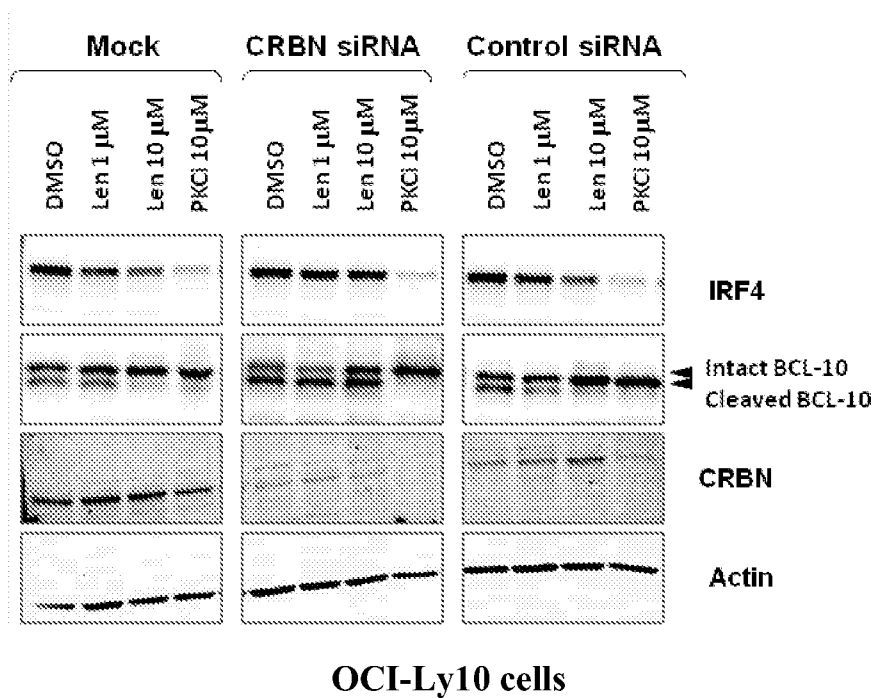
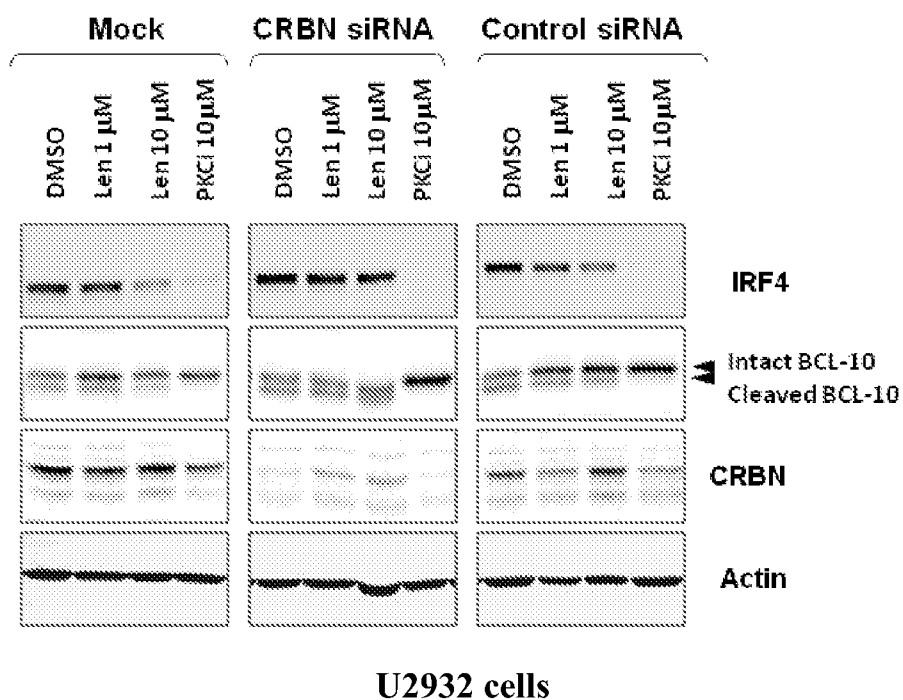
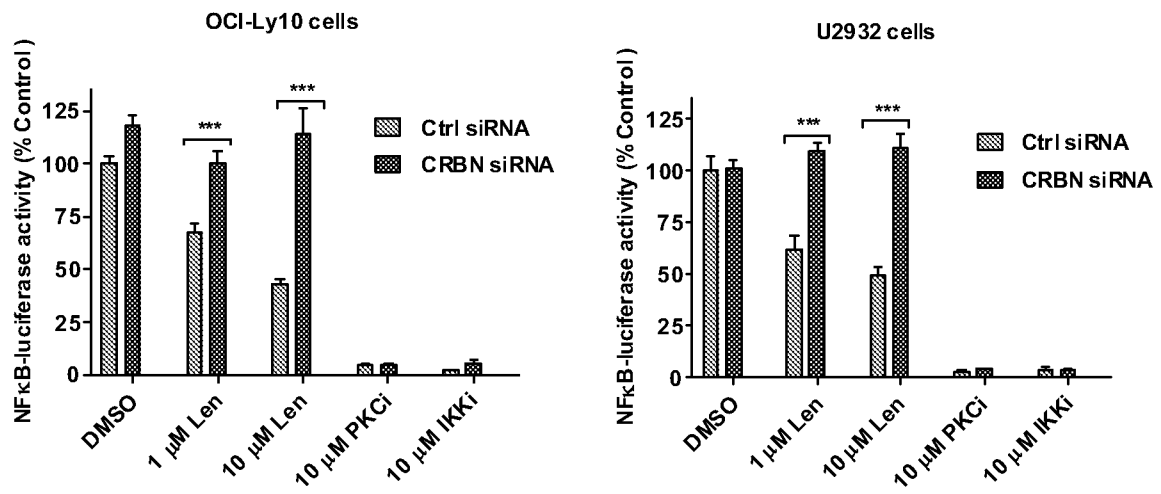


FIG 32C



Normalized to DMSO of control siRNA treated cells

FIG 32D

U2932 cells

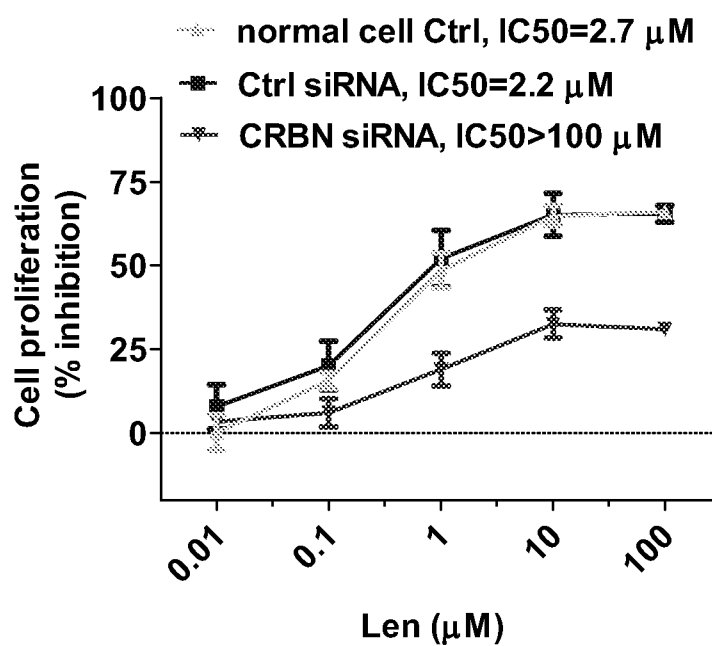
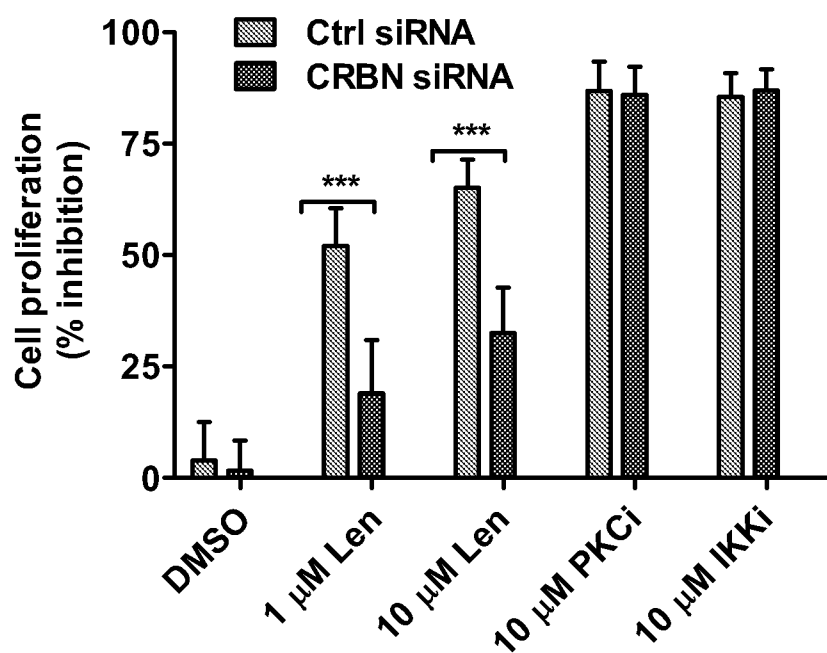


FIG 33A & 33B

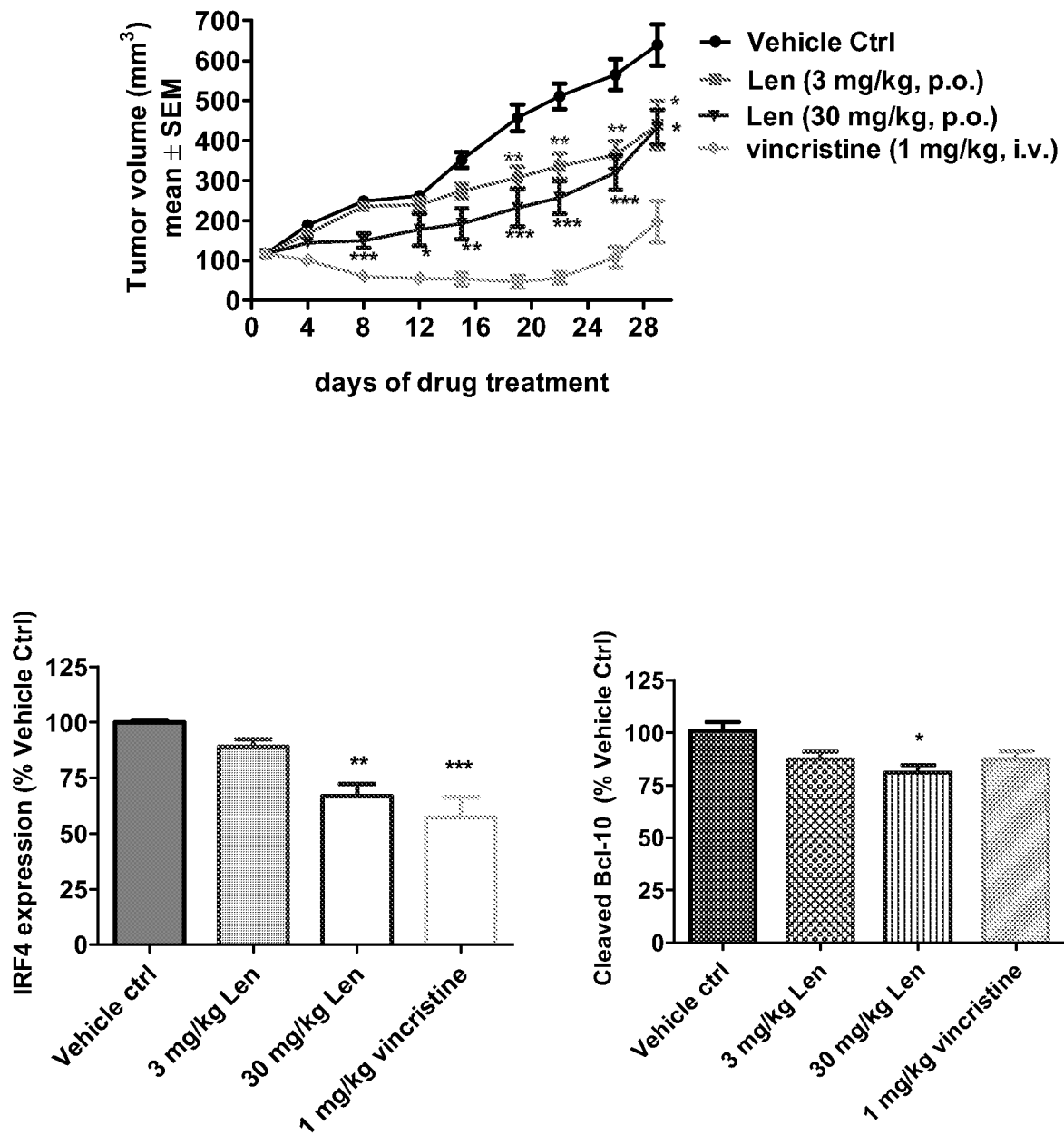


FIG 34A & 34B

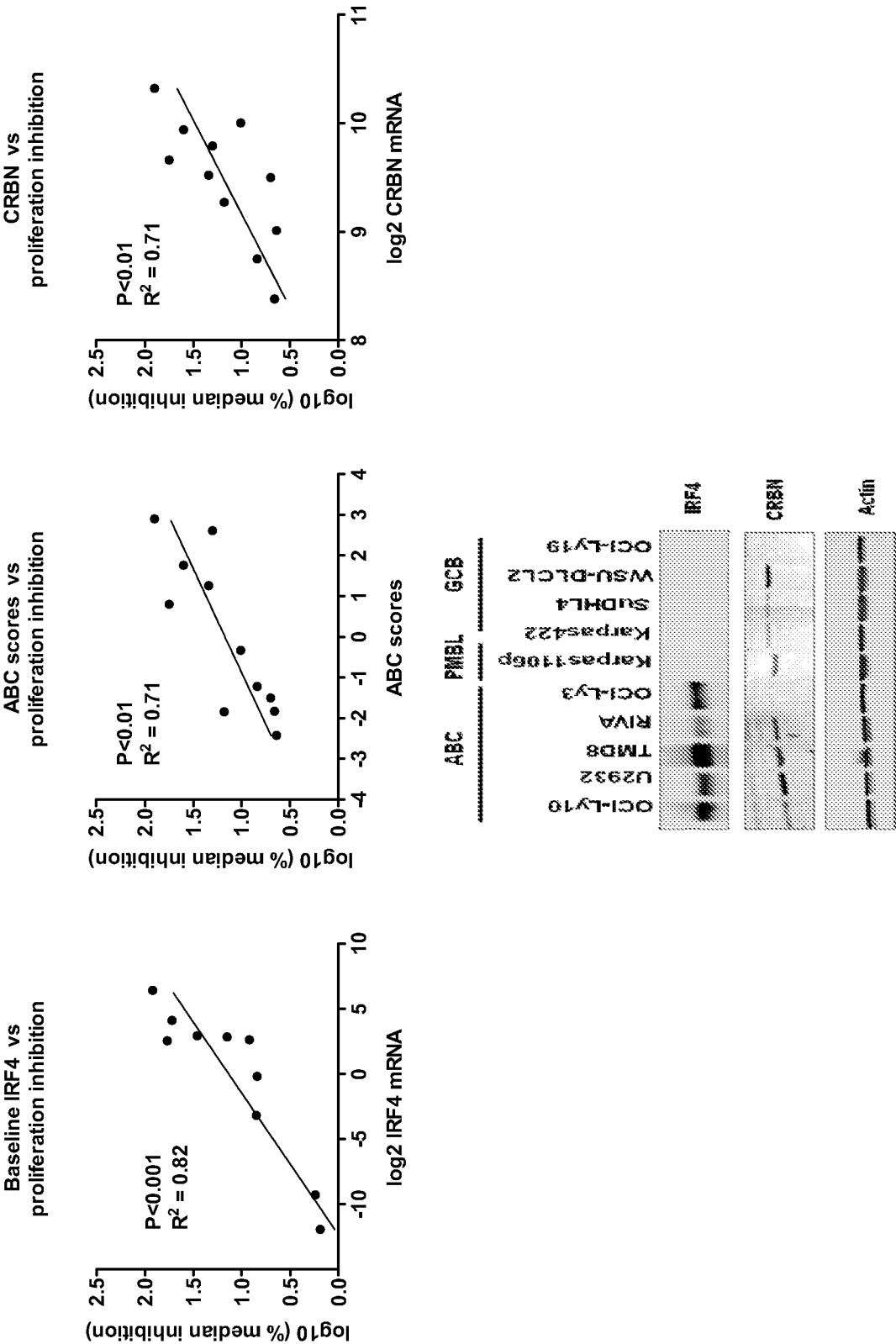


FIG. 35

CGN-6-1-11-HC CGN-6-4-5-HC	METGLRWLLLVAVLKGVCQSVEEGGRLVTPGTPLTLTCTVSGFSLSYGVSWVRQAPG METGLRWLLLVAVLKGVCQSVEEGGRLVTPGTPLTLTCTVSGFSLSYGVSWVRQAPG *****:***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	KGLEIYIYSDSDKTYATWAKGRFTISKTS-TTVDLKITSPTIEDTATYFCARGTPLA KLEHIGIYISDPGMYATWAKGRFTISKTSSTTVDLKMTSPTIEDTATYFCARGTPLA ***:*****..*:***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	SYSIWGPGTLVTVSLGQPKAPSVFPLAPCCGDTSPSTVTLGCLVKGYLPEPVTVTWNSGT SYSTWGPGLVTISLGQPKAPSVFPLAPCCGDTSPSTVTLGCLVKGYLPEPVTVTWNSGT *** *****:***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	LTNGVRTFPSVRQSSGLYSLSSVSVTSSSQPVTCNVVAPATNTKVDTKTVAPSTCSKPTC LTNGVRTFPSVRQSSGLYSLSSVSVTSSSQPVTCNVVAPATNTKVDTKTVAPSTCSKPTC ***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	PPPELLGGPSVFIFFPKPKDTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARP PPPELLGGPSVFIFFPKPKDTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARP ***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	PLREQQFNSTIRVVSTLPIAHQDWLRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVY PLREQQFNSTIRVVSTLPIAHQDWLRGKEFKCKVHNKALPAPIEKTISKARGQPLEPKVY ***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	TMGPPREELSSRSVSLTCMINGFYPSDISVEWEKNGKAEDNYKTTPAVLDSGSGYFLYSK TMGPPREELSSRSVSLTCMINGFYPSDISVEWEKNGKAEDNYKTTPAVLDSGSGYFLYSK ***** *****
CGN-6-1-11-HC CGN-6-4-5-HC	LSVPTSEWQRGDVFTCSVMHEALHNHYTQKSISRSPGK- LSVPTSEWQRGDVFTCSVMHEALHNHYTQKSISRSPGK- ***** *****

CGN-6-1-11-LC	MDRAPTQLLGLLLWLP	GATFAQVLTQTPASV	SAAGGTVTIN	CQASQSVYKNNYLSWF
CGN-6-4-5-LC	MDRAPTQLLGLLLWLP	GATFAQVLTQTPASV	SAAGGTVTIN	CQSSENIYKNNYLSWF
	*****	*****	*****	*****
	*****	*****	*****	*****
	*****	*****	*****	*****
CGN-6-1-11-LC	QKPGQPPKLLIYEAS	KLASGVPPRFKGS	GGTQFTFTISD	LECDDAAFYCAGGYGNI
CGN-6-4-5-LC	QKPGQPPKLLIYQAS	TLASGVPSRFKGS	GSGRFSLTISD	LECDDAATYYCAGGYGNI
	*****	*****	*****	*****
	*****	*****	*****	*****
	*****	*****	*****	*****
CGN-6-1-11-LC	FFFGGTEVVVKGD	PVAPTVLIFPPA	ADQVATGTVT	IVCVANKYFPDVTVTWEVDGTTQT
CGN-6-4-5-LC	FTFGGTEVVVKGD	PVAPTVLIFPPA	ADQVATGTVT	IVCVANKYFPDVTVTWEVDGTTQT
	*	*****	*****	*****
	*	*****	*****	*****
	*	*****	*****	*****
CGN-6-1-11-LC	TGIENSKTPQNSAD	CTYNLSSTLTLT	STQYN	SHKEYTCKVTQGTTSVVQSFN
CGN-6-4-5-LC	TGIENSKTPQNSAD	CTYNLSSTLTLT	STQYN	SHKEYTCKVTQGTTSVVQSFN
	*****	*****	*****	*****
	*****	*****	*****	*****
	*****	*****	*****	*****

FIG. 37

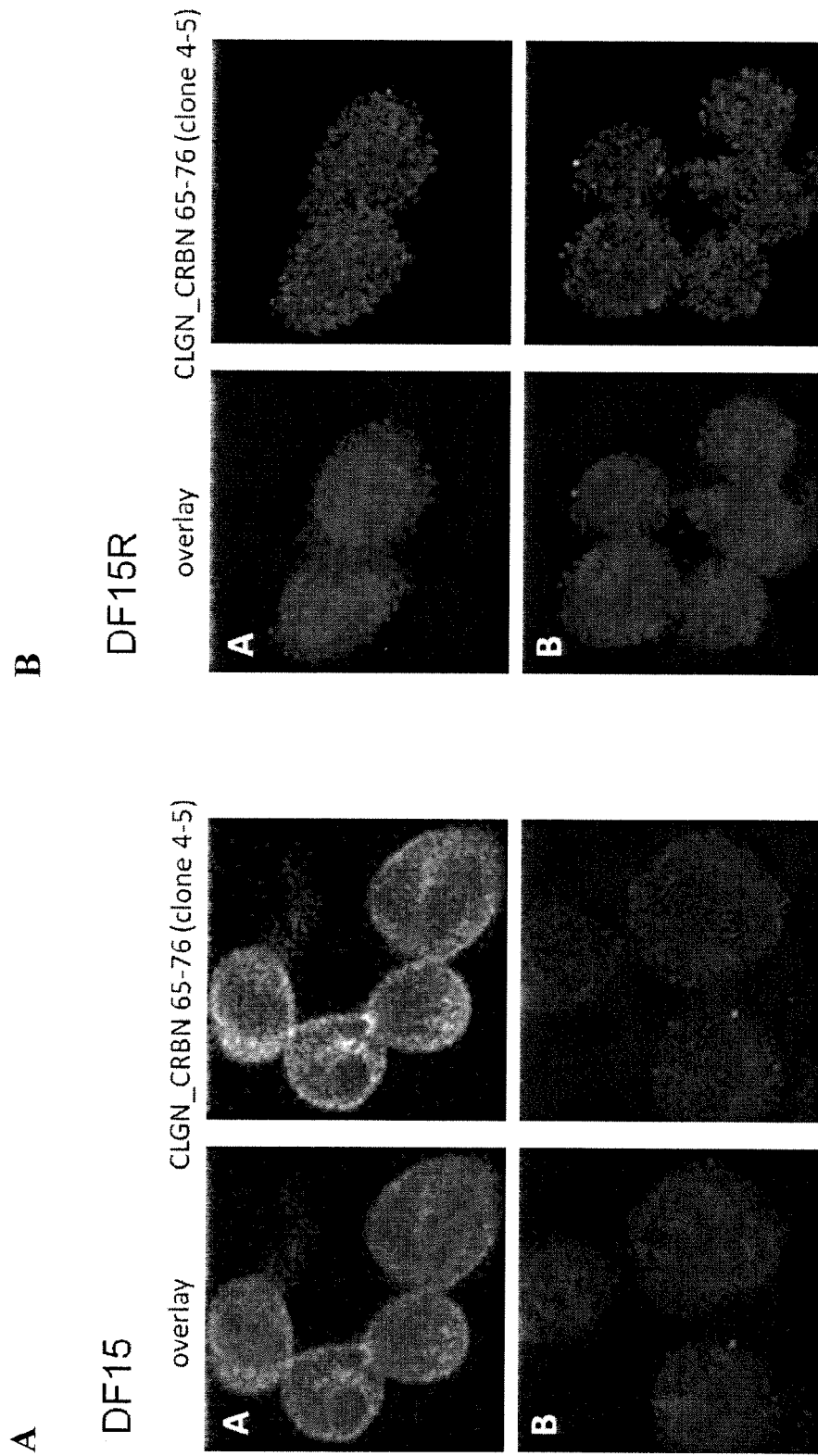


FIG. 39A

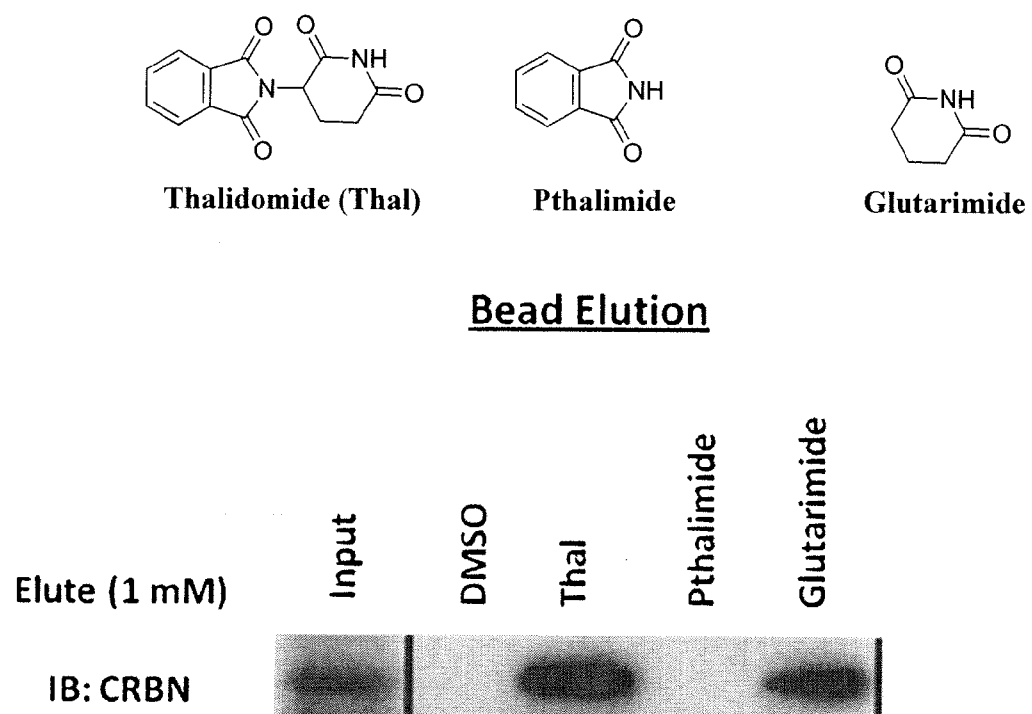
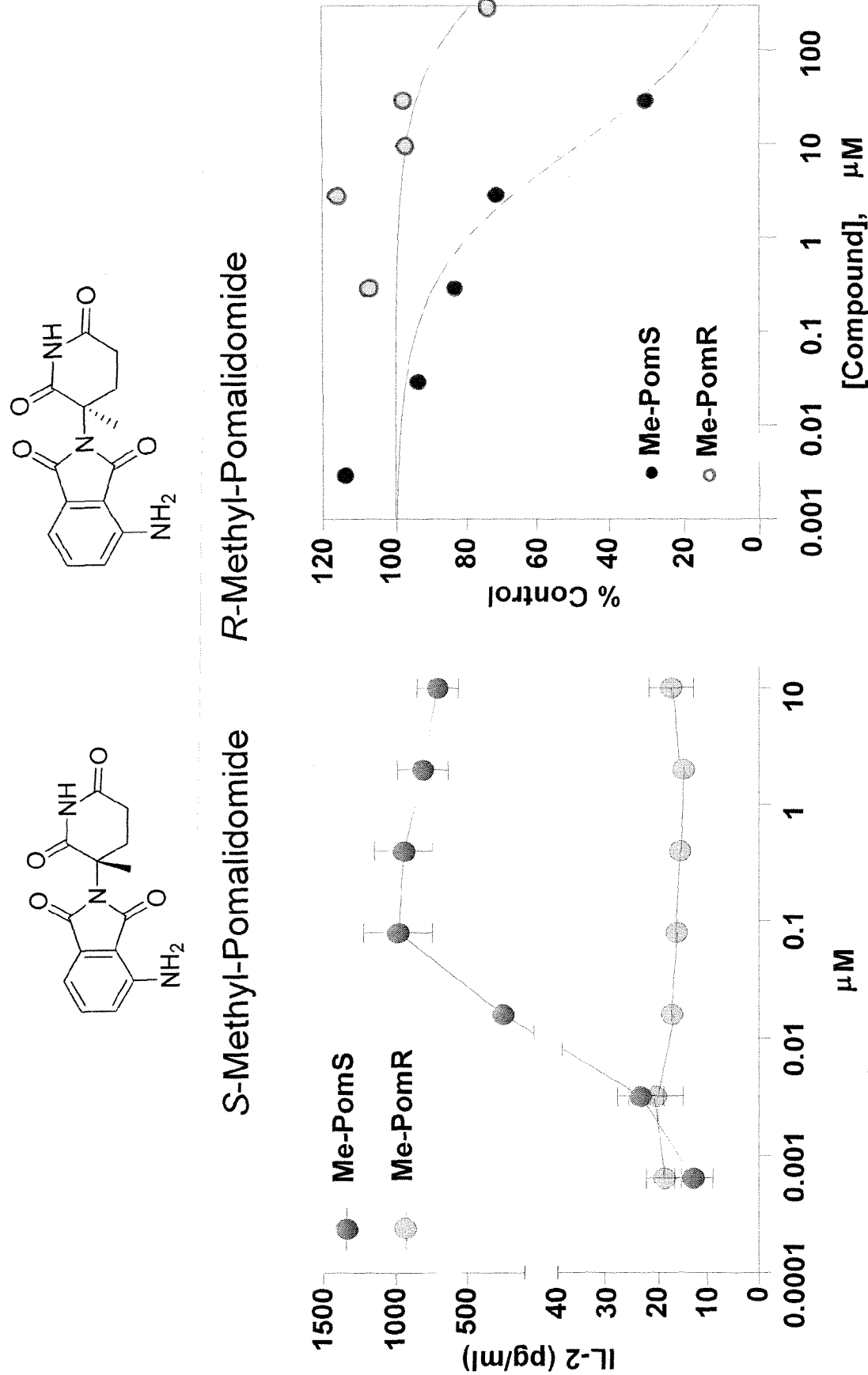


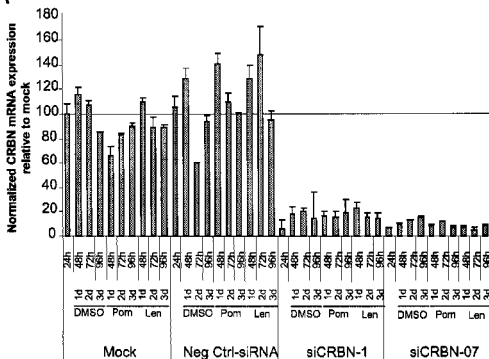
FIG. 39B



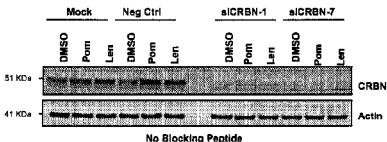
CRBN, cereblon; IL, interleukin; Me-PomR, R-methyl-pomalidomide; Me-PomS, S-methyl-pomalidomide.

FIG. 1A & 1B

A



B



C

