Abstract: The present disclosure pertains to methods, dosage forms, and kits for use in the treatment of multiple myeloma in a subject. For example, methods are provided for the treatment of multiple myeloma using a combination of a class I inhibitor of histone deacetylase, such as entinostat, and an antineoplastic alkylating agent, such as bendaustine. The combination of the class I inhibitor of histone deacetylase and the antineoplastic alkylating agent synergistically induces potent anti-proliferative and anti-survival effects in cancer cells, and represents a novel therapeutic approach for treating multiple myeloma, for example.

FIG. 1

A

B

U266

Survival (% Control)

0 50 100 150 200 400

Bevacizumab (μg/mL)

Survival (% Control)

0 50 100 150 200 400 600

Bevacizumab (μg/mL)

Survival (% Control)

0 50 100 150 200 400

Bevacizumab (μg/mL)

Survival (% Control)

0 50 100 150 200 400

Bevacizumab (μg/mL)
TREATMENT OF MULTIPLE MYELOMA

CROSS REFERENCE TO RELATED APPLICATIONS
This application claims the benefit of U.S. Provisional Application No. 61/618,548, filed March 30, 2012, and U.S. Provisional Application No. 61/726,296, filed November 14, 2012, the entireties of which are incorporated by reference herein.

TECHNICAL FIELD

[0001] The present disclosure pertains to methods and dosage forms for the treatment of multiple myeloma using pharmaceutical agents.

BACKGROUND

[0002] Cancer, tumors, tumor-related disorders, and neoplastic disease states are serious and often times life-threatening conditions. These diseases and disorders, which are characterized by rapidly proliferating cell growth, continue to be the subject of research efforts directed toward the identification of therapeutic agents which are effective in the treatment thereof. Such agents prolong the survival of the patient, inhibit the rapidly-proliferating cell growth associated with the neoplasm, or effect a regression of the neoplasm.

[0003] Generally, surgery and radiation therapy are the first modalities considered for the treatment of cancer that is considered locally confined, and offer the best prognosis. Chemotherapy treatment of certain cancers typically results in disappointing survival rates but still offer a survival benefit.

[0004] Histone deacetylase (HDAC) inhibitors are an emerging class of therapeutic agents that promote differentiation and apoptosis in hematologic and solid malignancies through chromatin remodeling and gene expression regulation. Several HDAC inhibitors have been identified including benzamides (entinostat), shortchain fatty acids (i.e., Sodium phenylbutyrate); hydroxamic acids (i.e., suberoylanilide hydroxamic acid and thrichostatin A); cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., trapoxin A) and cyclic peptides without the 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., FK228). Entinostat (Syndax Pharmaceuticals, Inc.) is a benzamide HDAC inhibitor undergoing clinical investigation in multiple types of solid tumors and hematologic cancers. Entinostat is rapidly absorbed and has a half-life of 100 hours; changes in histone acetylation have persisted for several weeks following the administration of entinostat.
There exists a pressing need for compositions and methods of treatment for cancers, such as multiple myeloma.

SUMMARY

Disclosed herein are methods of treating multiple myeloma in a patient comprising administering to the patient a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof.

The anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof may be administered prior to or following the administration of the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof, or may be coadministered with the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

In certain embodiments, the anti-neoplastic alkylating agent is bendamustine or a pharmaceutically acceptable salt thereof.

The class I inhibitor of histone deacetylase may be, for example, entinostat or a pharmaceutically acceptable salt thereof.

The anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof may be administered in a ratio of from about 250:1 to about 2500:1 relative to the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof. For example, the anti-neoplastic alkylating agent or pharmaceutically acceptable salt may be administered in a ratio of about 250, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1600, about 1700, about 1800, about 1900, about 2000, about 2100, about 2200, about 2300, about 2400, or about 2500 to 1 relative to the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

Also disclosed are pharmaceutical dosage forms comprising a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof. In certain embodiments of the present dosage forms, the anti-neoplastic alkylating agent is bendamustine or a pharmaceutically acceptable salt thereof. The class I inhibitor of histone deacetylase may be, for example, entinostat or a pharmaceutically acceptable salt thereof. In the disclosed dosage forms, the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof may be present in a ratio of from about 250:1 to about 2500:1 relative to the amount class I inhibitor of histone deacetylase or
pharmaceutically acceptable salt thereof that is present in the dosage form. For example, the anti-neoplastic alkylating agent or pharmaceutically acceptable salt may be present in a ratio of about 250, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1600, about 1700, about 1800, about 1900, about 2000, about 2100, about 2200, about 2300, about 2400, or about 2500 to 1 relative to the amount of class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

[0012] The present disclosure also pertains to kits that comprise at least one pharmaceutical dosage form comprising a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and at least one pharmaceutical dosage form comprising a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof. The at least one pharmaceutical dosage form that comprises a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof may comprise, for example, bendamustine. The at least one pharmaceutical dosage form that comprises a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof may comprise, for example, entinostat. The ratio of the amount of anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof in a dosage form of the presently disclosed kits may be from about 250:1 to about 2500:1 relative to the amount of class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof in another dosage forms in the present kits. For example, the anti-neoplastic alkylating agent or pharmaceutically acceptable salt may be present in a ratio of about 250, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1600, about 1700, about 1800, about 1900, about 2000, about 2100, about 2200, about 2300, about 2400, or about 2500 to 1 relative to the amount of class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0013] FIG. 1 depicts the results of a study that demonstrated that bendamustine or entinostat alone inhibits proliferation of MM cells in a dose-dependent manner. Human MM cells were plated onto 96-well plates with fresh RPMI1640 medium (0.5% FBS) or same medium containing indicated concentrations of bendamustine or entinostat for 72 h. The percentages of surviving cells as compared to controls, defined as 100% survival, were
determined by reduction of MTS. Data shows the representative of three independent experiments. Bars, SD. A, bendamustine; B, entinostat.

[0014] FIG. 2 depicts the results of a study demonstrating that the combination of bendamustine and entinostat significantly induces growth inhibition of MM cells, and is synergistic over a wide range of effects. (A) Human MM cells were plated onto 96-well plates with fresh RPMI1640 medium (0.5% FBS) or same medium containing indicated concentrations of bendamustine or entinostat or their combinations with a fixed ratio for 72 h. The percentages of surviving cells as compared to controls, defined as 100% survival, were determined by reduction of MTS. Data shows the representative of three independent experiments. Bars, SD. P values vs bendamustine single agent. (B) The combination index (CI) curves were calculated using CalcuSyn software according to the Chou-Talalay equation.

[0015] FIG. 3 provides the results of a study demonstrating that the combination of bendamustine and entinostat significantly promotes MM cells undergoing apoptosis. MM cells were cultured with RPMI1640 (0.5% FBS) in the absence or presence of entinostat, bendamustine alone or the combinations of entinostat and bendamustine for 24 h. Cells were collected and subjected to apoptotic ELISA (A) or western blot analyses with specific antibody directed against PARP, caspase-8 (Casp-8), caspase-3 (Casp-3), or β-actin (B, C, D). Bars, SD. P values versus bendamustine single agent.

[0016] FIG. 4 concerns the results of a study demonstrating that the combination of bendamustine and entinostat enhances DNA damage response. MM cells were cultured with RPMI1640 (0.5% FBS) in the absence or presence of entinostat, bendamustine alone or the combinations of entinostat and bendamustine for 24 h. Cells were collected and subjected to western blot analyses with specific antibody directed against P-H2AX, H2AX, P-CHK1, CHK1, P-CHK2, CHK2 or β-actin. A, U266; B, MM1.S; C, MM1.R.

[0017] FIG. 5 concerns the results of a study demonstrating that the combination of bendamustine and entinostat induces mitotic catastrophe. (A) MM1.S cells were cultured with RPMI1640 (0.5% FBS) in the absence or presence of entinostat, bendamustine alone or the combinations of entinostat and bendamustine for 24 h. Cells were collected and subjected to cytopsin onto cell slides followed by Giemsa staining and examination under a photomicroscope (x40 magnification). Arrows indicate the cells with mitotic catastrophe. (B) Cells that showed atypical mitotic figures, multi-nucleation, atypical chromosome clusters, and/or apoptosis were counted against normal cells, and reported in percentage. Data shows the representative of three independent experiments. Bars, SD. P values vs bendamustine single agent.
DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0018] The presently disclosed inventions may be understood more readily by reference to the following detailed description taken in connection with the accompanying figures and examples, which form a part of this disclosure. It is to be understood that these inventions are not limited to the specific products, methods, conditions or parameters described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed inventions.

[0019] In the present disclosure the singular forms "a," "an," and "the" include the plural reference, and reference to a particular numerical value includes at least that particular value, unless the context clearly indicates otherwise. Thus, for example, a reference to "an antioxidant" may be a reference to one or more of such substances and equivalents thereof known to those skilled in the art, and so forth. When values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. As used herein, "about X" (where X is a numerical value) preferably refers to ±10% of the recited value, inclusive. For example, the phrase "about 8" preferably refers to a value of 7.2 to 8.8, inclusive; as another example, the phrase "about 8%" preferably (but not always) refers to a value of 7.2% to 8.8%, inclusive. Where present, all ranges are inclusive and combinable. For example, when a range of "1 to 5" is recited, the recited range should be construed as including ranges "1 to 4", "1 to 3", "1-2", "1-2 & 4-5", "1-3 & 5", "2-5", and the like. In addition, when a list of alternatives is positively provided, such listing can be interpreted to mean that any of the alternatives may be excluded, e.g., by a negative limitation in the claims. For example, when a range of "1 to 5" is recited, the recited range may be construed as including situations whereby any of 1, 2, 3, 4, or 5 are negatively excluded; thus, a recitation of "1 to 5" may be construed as including the option of specifying "1 and 3-5, but not 2", or simply "wherein 2 is not included." It is intended that any component, element, attribute, or step that is positively recited herein may be explicitly excluded in the claims, whether such components, elements, attributes, or steps are listed as alternatives or whether they are recited in isolation.

[0020] Unless otherwise specified, any component, element, attribute, or step that is disclosed with respect to one embodiment of the present methods and products may apply to any other method or product that is disclosed herein.

[0021] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.
Bendamustine, a hybrid molecule of purine analog and alkylator, induces cell death by activation of apoptosis and DNA damage response and induction of mitotic catastrophe. Entinostat, a selective class I inhibitor of HDAC, exerts anti-tumor activity in a variety of cancer types, including multiple myeloma (MM). Cell growth assays performed by the present inventors and described more fully herein demonstrated that bendamustine or entinostat alone can inhibit cell proliferation in a dose-dependent manner. However, the present disclosure pertains to the surprising discovery that the combination of the class I inhibitor of HDAC and the anti-neoplastic alkylating agent synergistically inhibit growth and survival in several different multiple myeloma cell lines. For example, an apoptotic-ELISA and western blot analyses on PARP cleavage and activation of caspase-8 and caspase-3 revealed that the combinations of bendamustine and entinostat exhibited a much more potent activity than either agent alone to promote the MM cells undergoing apoptosis in a dose-dependent manner. Furthermore, studies on DNA damage response indicated that phospho-histone H2A.X (P-H2A.X), a hall marker of DNA double strand break, along with the phosphorylated CHK2 (P-CHK2) was significantly enhanced by the combinations of bendamustine and entinostat as compared to either agent alone. These molecular changes were correlated well with the increases in mitotic catastrophe. Collectively, these discoveries demonstrate that anti-neoplastic alkylating agents such as bendamustine in combination with class I inhibitors of histone deacetylase such as entinostat exhibit potent anti-proliferative/anti-survival activity in multiple myeloma cells via induction of apoptosis and DNA damage response.

Multiple myeloma (MM) is a plasma-cell neoplasm which is characterized by clonal proliferation of malignant plasma cells and the symptoms of skeletal destruction, renal impairment, and hematological dysfunctions [1]. Despite recent progress in understanding the biology of MM and developing novel agents and strategies, the prognosis of most MM patients is still poor, and resistance to traditional chemotherapy occurs frequently. A number of investigators have focused on studying the aberrant signaling pathways in the pathogenesis of MM and identifying abnormal protein expression involved in these pathways. Such study gives promise to targeted therapy and drug combination to overcome resistance [2].

Bendamustine was first synthesized in the 1960s in Germany. Similar to but not the same as other alkylating agents, bendamustine combines the alkylating activity of the mustard group with the anti-metabolite activity of the purine analog structure, which makes it have a unique pharmacological profile. It has been reported that bendamustine has long-lasting DNA damage action, and can induce apoptosis and mitotic catastrophe and inhibit mitotic checkpoint. In addition, it does not show cross-resistance with other cytotoxic agents [3].
preclinical studies, bendamustine is able to overcome resistance to other alkylating agents [4], and shows synergistic inhibitory effects with cladribine or rituximab on lymphoma cells or xenograft models [5; 6]. Recent clinical trials have shown that bendamustine combined with first line agents is safe and effective in the treatment of relapsed and/or refractory chronic lymphocytic leukemia (CML) [7], lymphoma [8; 9], and MM [10; 11].

[0025] Histone acetylation is a major epigenetic modification which regulates gene expression and affects tumorigenesis in various human cancers, including MM. It is controlled by the balance between histone deacetylase (HDAC) and histone acetyltransferase (HAT) [12]. HDACs modulate many oncogenes and tumor suppressor genes instead of directly affecting oncogenesis [13]. In addition, the function of many nonhistone proteins is also controlled by HDACs. In cancer cells, HDACs regulate many biological functions including proliferation, differentiation, apoptosis and survival by modulating multiple factors in signaling pathways. Furthermore, higher levels of histone acetylation are found in normal tissues as compared to tumors, and HDACs are typically overexpressed in tumor cells. Several HDAC inhibitors (HDACi) have been designed to target different types of HDACs [14]. Entinostat (also known as SNDX-275 or MS-275) is a synthetic benzamide derivative which inhibits class I HDACs. In vitro or in vivo studies have demonstrated that entinostat exhibits anti-tumor activity in multiple solid tumors or hematological malignancies [15]. Previous studies conducted by the present inventors demonstrated that entinostat induces apoptosis via down-regulation of erbB3 expression, enhances efficacy of trastuzumab, and has potential to overcome trastuzumab resistance in erbB2-overexpressing breast cancer cells [16; 17]. The present inventors also found that entinostat in combination with melphalan synergistically enhances DNA damage response and apoptosis in MM cells [18].

[0026] To facilitate understanding of the disclosure set forth herein, a number of terms are defined below.

[0027] As used herein, "abnormal cell growth," refers to cell growth that is independent of normal regulatory mechanisms (e.g., loss of contact inhibition), including the abnormal growth of normal cells and the growth of abnormal cells.

[0028] "Neoplasia" as described herein, is an abnormal, unregulated and disorganized proliferation of cells that is distinguished from normal cells by autonomous growth and somatic mutations. As neoplastic cells grow and divide they pass on their genetic mutations and proliferative characteristics to progeny cells. A neoplasm, or tumor, is an accumulation of neoplastic cells. In some embodiments, the neoplasm can be benign or malignant.
"Metastasis," as used herein, refers to the dissemination of tumor cells via lymphatics or blood vessels. Metastasis also refers to the migration of tumor cells by direct extension through serous cavities, or subarachnoid or other spaces. Through the process of metastasis, tumor cell migration to other areas of the body establishes neoplasms in areas away from the site of initial appearance.

As discussed herein, "angiogenesis" is prominent in tumor formation and metastasis. Angiogenic factors have been found associated with several solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is important include solid tumors such as renal cell carcinoma, hepatocellular carcinoma, and benign tumors such as acoustic neuroma, and neurofibroma. Angiogenesis has been associated with bloodborn tumors such as leukemias. It is believed that angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia. Prevention of angiogenesis could halt the growth of cancerous tumors and the resultant damage to the subject due to the presence of the tumor.

The term "subject" refers to an animal, including, but not limited to, a primate (e.g., human), cow, sheep, goat, horse, dog, cat, rabbit, rat, or mouse. The terms "subject" and "patient" are used interchangeably herein in reference, for example, to a mammalian subject, such as a human subject.

The terms "treat," "treating," and "treatment" are meant to include alleviating or abrogating a disorder, disease, or condition; or one or more of the symptoms associated with the disorder, disease, or condition; or alleviating or eradicating the cause(s) of the disorder, disease, or condition itself.

The term "therapeutically effective amount" refers to the amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the symptoms of the disorder, disease, or condition being treated. The term "therapeutically effective amount" also refers to the amount of a compound that is sufficient to elicit the biological or medical response of a cell, tissue, system, animal, or human that is being sought by a researcher, veterinarian, medical doctor, or clinician.

The term "pharmaceutically acceptable carrier," "pharmaceutically acceptable excipient," "physiologically acceptable carrier," or "physiologically acceptable excipient" refers to a pharmaceutically-acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, or encapsulating material. Each component must be "pharmaceutically acceptable" in the sense of being compatible with the other ingredients of a

[0035] The term "pharmaceutical composition" refers to a mixture of a compound disclosed herein with other chemical components, such as diluents or carriers. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds with inorganic or organic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

[0036] Compounds of the present invention may take the form of a pharmacologically acceptable salt, hydrate, or solvate. Pharmacologically acceptable salts include basic salts of inorganic and organic acids, including but not limited to hydrochloric acid, hydrobromic acid, sulphuric acid, phosphoric acid, methanesulphonic acid, ethanesulfonic acid, malic acid, acetic acid, oxalic acid, tartaric acid, citric acid, lactic acid, fumaric acid, succinic acid, maleic acid, salicylic acid, benzoic acid, phenylacetic acid, mandelic acid and the like. When compounds of the invention include an acidic function, such as a carboxyl group, then suitable pharmaceutically acceptable cation pairs for the carboxyl group are well known to those skilled in the art and include alkaline, alkaline earth, ammonium, quaternary ammonium cations and the like.

[0037] Histone Deacetylase. The HDACs are a family including at least eighteen enzymes, grouped in three classes (Class I, II and III). Class I HDACs include, but are not limited to, HADCs 1, 2, 3, and 8. Class I HDACs can be found in the nucleus and are believed to be involved with transcriptional control repressors. Class II HDACs include, but are not limited to, HDACS 4, 5, 6, 7, and 9 and can be found in both the cytoplasm as well as the nucleus. Class III HDACs are believed to be NAD dependent proteins and include, but are not limited to, members of the Sirtuin family of proteins. Non-limiting examples of sirtuin proteins include
SIRT1-7. As used herein, the term "selective HDAC" refers to an HDAC inhibitor that does not interact with all three HDAC classes.

[0038] **HDAC Inhibitors.** HDAC inhibitors can be classified broadly into pan HDAC inhibitors and selective HDAC inhibitors. Although there is a large structural diversity of known HDAC inhibitors, they share common features: a part that interacts with the enzyme active site and a side-chain that sits inside the channel leading to the active site. This can be seen with the hydroxamates such as SAHA, where the hydroxamate group is believed to interact with the active site. In the case of the depsipeptides, it is believed that an intracellular reduction of the disulphide bond creates a free thiol group (which interacts with the active site) attached to a 4-carbon alkenyl chain. A difference between the HDAC inhibitors is in the way that they interact with the rim of the HDAC channel, which is at the opposite end of the channel to the active site. It is this interaction, between the HDAC inhibitor and the rim of the channel, which is believed to account, at least in part, for some observed differences in HDAC selectivity between pan-HDAC inhibitors, such as SAHA and selective HDAC inhibitors such as the depsipeptides. A particularly preferred HDAC inhibitor is entinostat. Entinostat has the chemical name N-(2-aminophenyl)-4-[N-(pyridine-3-yl)methoxycarbonylamino-methyl]-benzamide and the chemical structure shown below:

![Entinostat](image)

[0039] **Bendamustine** (Cephalon, Inc., West Chester, PA), a hybrid molecule of purine analog and alkylator, induces cell death by activation of DNA damage response and apoptosis, inhibition of mitotic checkpoints, and induction of mitotic catastrophe. Bendamustine hydrochloride is marketed in the United States as Treanda® for the treatment of chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, and multiple myeloma. The chemical structure of bendamustine is shown below:
[0040] Bendamustine, or a pharmaceutically acceptable salt thereof, can be provided in a variety of pharmaceutically acceptable dosage forms. One particularly preferred form is a lyophilized pharmaceutical composition. Lyophilized pharmaceutical compositions of bendamustine can be prepared according to methods known to those skilled in the art. An exemplary method for preparing a lyophilized pharmaceutical composition comprising bendamustine is described in U.S. Published Application No. 2006/0159713, the entirety of which is incorporated herein.

[0041] The lyophilized pharmaceuticals disclosed herein can be provided in a container and in an amount suitable for reconstitution and delivery of one or more doses, typically about 1-2, 1-3, 1-4, 1-5, 1-6, 1-10, or about 1-20 doses. The pharmaceutical composition suitable for injection or infusion use can include sterile aqueous solutions or dispersions or sterile powders comprising an active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The prevention of the growth of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

[0029] Bendamustine, or a pharmaceutically acceptable salt thereof, can also be provided as a ready-to-use liquid formulation. Such exemplary formulations are described in U.S. Published Application No. 2012/0129904.

[0042] Additional Therapies. Available additional treatments for multiple myeloma that may be advantageously employed in combination with the therapies disclosed herein include, without limitation, radiation therapy, chemotherapy, antibody therapy, and tyrosine kinase inhibitors as adjuvant therapy.

[0043] Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation to kill cancer cells or keep them from growing. Chemotherapy is a cancer
treatment that uses drugs to stop the growth of cancer cells, either by killing the cells or by stopping them from dividing. When chemotherapy is taken by mouth or injected into a vein or muscle, the drugs enter the bloodstream and can reach cancer cells throughout the body (systemic chemotherapy). When chemotherapy is placed directly into the spinal column, an organ, or a body cavity such as the abdomen, the drugs mainly affect cancer cells in those areas (regional chemotherapy). The way the chemotherapy is given depends on the type and stage of the cancer being treated.

Different chemotherapeutic agents are known in the art for treating multiple myeloma. Cytotoxic agents used for treating multiple myeloma include doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracil, mitomycin C, mitoxantrone, paclitaxel, taxane formulations such as by way of example only, Abraxane® (ABI-007), Paclitaxel-Cremophor EL, Paclitaxel poliglumex, and Paclitaxel injectable emulsion (PIE), gemcitabine, docetaxel, capecitabine and epirubicin.

Other chemotherapy against multiple myeloma includes treatment with one or more of carboplatin (for example, Paraplatin®), carmustine (for example, BCNU®), chlorambucil (for example, Leukeran®), cisplatin (for example, Platinol®), cyclophosphamide injection (for example, Cytoxan®), oral cyclophosphamide (for example, Cytoxan®), dacarbazine (for example, DTIC®), ifosfamide (for example, ifex®), lomustine (for example, CCNU®), mechlorethamine (for example, nitrogen mustard, Mustargen®), melphalan (for example, Alkeran®), procarbazine (for example, Matulane®), bleomycin (for example, Blenoxane®), doxorubicin (for example, Adriamycin®, Rubex®), epirubicin, Idarubicin (for example, Idamycin®), mitoxantrone (for example, Novantrone®), gemcitabine (for example, Gemzar®), oral mercaptopurine (for example, Purinethol®), methotrexate, pentostatin IV (for example, Nipent®), oral thioguanine (for example, Lanvis®), oral etoposide (for example, VP-16, VePesid®, Etopophos) - etoposide IV (for example, VP-16, VePesid®, Etopophosph), vinblastine (for example, Velban®, vincristine (for example, Oncovin®), vinorelbine (for example, Navelbine®), dexamethasone (for example, Decadron®), methylprednisolone (for example, Medrol®), and prednisone (for example, Deltasone®).

Monoclonal antibody therapy is a cancer treatment that uses antibodies made in the laboratory, from a single type of immune system cell. These antibodies can identify substances on cancer cells or normal substances that may help cancer cells grow. The antibodies attach to the substances and kill the cancer cells, block their growth, or keep them from spreading. Monoclonal antibodies are given by infusion. They may be used alone or to carry
drugs, toxins, or radioactive material directly to cancer cells. Monoclonal antibodies are also used in combination with chemotherapy as adjuvant therapy.

[0047] Additional, illustrative, treatments that may be advantageously combined with the compositions and therapies disclosed herein may include, without limitation, administration of agents including, but not limited to lapatinib, alone or in combination with capecitabine, docetaxel, epirubicin, epothilone A, B or D, goserelin acetate, paclitaxel, pamidronate, bevacizumab, or trastuzumab.

[0048] In some embodiments, the additional therapy comprises chemotherapy comprising administering to the subject one or more of doxorubicin, cyclophosphamide, paclitaxel, lapatinib, capecitabine, trastuzumab, bevacizumab, gemcitabine, eribulin, or nab-paclitaxel.

[0049] Pursuant to the present methods, the class I inhibitor of histone deacetylase and the anti-neoplastic alkylating agent may respectively be administered by any therapeutically acceptable route. For example, the agents may be delivered orally, intravenously, subcutaneously, through inhalation, topically, enterally, parenterally, gastronomically, or by any other suitable route. The agents may also be administered by respectively different routes. This may be the case whether the class I inhibitor of histone deacetylase and the anti-neoplastic alkylating agent are coadministered or are administered at different times relative to one another. For example, in such embodiments, one of the agents is administered orally whereas the other agent is administered intravenously, and these administrations may occur substantially simultaneously or at different times relative to one another.

[0050] In the presently disclosed kits, both the dosage form that comprises a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and the dosage form that comprises a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof may respectively be any dosage form that permits administration of drug by a therapeutically acceptable route. For example, the respective dosage forms may be configured for delivery of the agents orally, intravenously, subcutaneously, through inhalation, topically, enterally, parenterally, gastronomically, or by any other suitable route.

[0051] Some embodiments of the present kits comprise at least two dosage forms that each comprise a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof. Such dosage forms may respectively contain equal or different quantities of the active agent, and may respectively be configured for delivering the active agent via the same or different routes. For example, the kits may comprise two or more
dosage forms, wherein each comprise the same amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and each is configured for delivering the drug via the same route. In another embodiment, the kits comprise two or more dosage forms that respectively comprise different amounts of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof. The same anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof may be used in each of the dosage forms of the kit, or may be different. In one embodiment, the anti-neoplastic alkylating agent in each of the dosage forms is bendamustine or a pharmaceutically acceptable salt thereof.

[0052] Also disclosed are embodiments of the present kits that comprise at least two dosage forms that each comprise a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof. Such dosage forms may respectively contain equal or different quantities of the active agent, and may respectively be configured for delivering the active agent via the same or different routes. For example, the kits may comprise two or more dosage forms, wherein each comprise the same amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof, and each is configured for delivering the drug via the same route. In another embodiment, the kits comprise two or more dosage forms that respectively comprise different amounts of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof. The same class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof may be used in each of the dosage forms of the kit, or may be different. In one embodiment, the class I inhibitor of histone deacetylase in each of the dosage forms is entinostat or a pharmaceutically acceptable salt thereof.

[0053] Oral formulations containing the active pharmaceutical ingredients described herein may comprise any conventionally used oral forms, including: tablets, capsules, pills, troches, lozenges, astilles, cachets, pellets, medicated chewing gum, granules, bulk powders, effervescent or non-effervescent powders or granules, solutions, emulsions, suspensions, solutions, wafers, sprinkles, elixirs, syrups, buccal forms, and oral liquids. Capsules may contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (e.g., corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium
lauryl sulfate, microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. In some embodiments are surface modifying agents which include nonionic and anionic surface modifying agents. For example, surface modifying agents include, but are not limited to, starches, such as corn starch, potato starch, and pre-gelatinized starch (e.g., STARCH 1500); pellets, provided time may mean colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

[0054] As indicated above, pursuant to the present methods, the respective drugs may be administered substantially simultaneously (co-administered) or can be administered in a staggered regimen, with the class I inhibitor of histone deacetylase (such as entinostat) being given at a different time than the antineoplastic alkylating agent, such as bendamustine. This time differential may range from several minutes, hours, days, weeks, or longer between administrations of the two compounds. Therefore, the term "combination" does not necessarily mean administered at precisely the same time or as a unitary dose, but that each of the components are administered during a desired treatment period. As indicated above, the agents may also be administered by respectively different routes.

[0055] In other embodiments, the pharmaceutical compositions provided herein may be provided in solid, semisolid, or liquid dosage forms for oral administration. As used herein, oral administration also include buccal, lingual, and sublingual administration. Suitable oral dosage forms include, but are not limited to, tablets, capsules, pills, troches, lozenges, pastilles, cachets, pellets, medicated chewing gum, granules, bulk powders, effervescent or non-effervescent powders or granules, solutions, emulsions, suspensions, solutions, wafers, sprinkles, elixirs, and syrups. In addition to the active ingredient(s), the pharmaceutical compositions may contain one or more pharmaceutically acceptable carriers or excipients, including, but not limited to, binders, fillers, diluents, disintegrants, wetting agents, lubricants, glidants, coloring agents, dye-migration inhibitors, sweetening agents, and flavoring agents.

[0056] Binders or granulators impart cohesiveness to a tablet to ensure the tablet remaining intact after compression. Suitable binders or granulators include, but are not limited to, starches, such as corn starch, potato starch, and pre-gelatinized starch (e.g., STARCH 1500);
gelatin; sugars, such as sucrose, glucose, dextrose, molasses, and lactose; natural and synthetic
gums, such as acacia, alginic acid, alginates, extract of Irish moss, Panwar gum, ghatti gum,
mucilage of isabgol husks, carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone
(PVP), Veegum, larch arabogalactan, powdered tragacanth, and guar gum; cellulosics, such as
ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl
cellulose, methyl cellulose, hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC),
hydroxypropyl methyl cellulose (HPMC); microcrystalline cellulosics, such as AVICEL-PH-101,
AVICEL-PH-103, AVICEL RC-581, AVICEL-PH-105 (FMC Corp., Marcus Hook, PA); and
mixtures thereof. Suitable fillers include, but are not limited to, talc, calcium carbonate,
microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol,
starch, pre-gelatinized starch, and mixtures thereof. The binder or filler may be present from
about 50 to about 99% by weight in the pharmaceutical compositions provided herein.

[0057] Suitable diluents include, but are not limited to, dicalcium phosphate, calcium
sulfate, lactose, sorbitol, sucrose, inositol, cellulose, kaolin, mannitol, sodium chloride, dry
starch, and powdered sugar. Certain diluents, such as mannitol, lactose, sorbitol, sucrose, and
inositol, when present in sufficient quantity, can impart properties to some compressed tablets
that permit disintegration in the mouth by chewing. Such compressed tablets can be used as
chewable tablets.

[0058] Suitable disintegrants include, but are not limited to, agar; bentonite; cellulosics,
such as methylcellulose and carboxymethylcellulose; wood products; natural sponge; cation-
exchange resins; alginic acid; gums, such as guar gum and Veegum HV; citrus pulp; cross-linked
cellulosics, such as croscarmellose; cross-linked polymers, such as crospovidone; cross-linked
starches; calcium carbonate; microcrystalline cellulose, such as sodium starch glycolate;
polacrilin potassium; starches, such as corn starch, potato starch, tapioca starch, and pre-
gelatinized starch; clays; aligns; and mixtures thereof. The amount of disintegrant in the
pharmaceutical compositions provided herein varies upon the type of formulation, and is readily
discernible to those of ordinary skill in the art. The pharmaceutical compositions provided herein
may contain from about 0.5 to about 15% or from about 1 to about 5% by weight of a
disintegrant.

[0059] Suitable lubricants include, but are not limited to, calcium stearate; magnesium
stearate; mineral oil; light mineral oil; glycerin; sorbitol; mannitol; glycols, such as glycerol
behenate and polyethylene glycol (PEG); stearic acid; sodium lauryl sulfate; t alc; hydrogenated
vegetable oil, including peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil,
and soybean oil; zinc stearate; ethyl oleate; ethyl laureate; agar; starch; lycopodium; silica or
silica gels, such as AEROSIL® 200 (W.R. Grace Co., Baltimore, MD) and CAB-O-SIL® (Cabot Co. of Boston, MA); and mixtures thereof. The pharmaceutical compositions provided herein may contain about 0.1 to about 5% by weight of a lubricant.

[0060] Suitable glidants include colloidal silicon dioxide, CAB-O-SIL® (Cabot Co. of Boston, MA), and asbestos-free talc. Coloring agents include any of the approved, certified, water soluble FD&C dyes, and water insoluble FD&C dyes suspended on alumina hydrate, and color lakes and mixtures thereof. A color lake is the combination by adsorption of a water-soluble dye to a hydrous oxide of a heavy metal, resulting in an insoluble form of the dye. Flavoring agents include natural flavors extracted from plants, such as fruits, and synthetic blends of compounds which produce a pleasant taste sensation, such as peppermint and methyl salicylate. Sweetening agents include sucrose, lactose, mannitol, syrups, glycerin, and artificial sweeteners, such as saccharin and aspartame. Suitable emulsifying agents include gelatin, acacia, tragacanth, bentonite, and surfactants, such as polyoxyethylene sorbitan monooleate (TWEEN® 20), polyoxyethylene sorbitan monooleate 80 (TWEEN® 80), and triethanolamine oleate. Suspending and dispersing agents include sodium carboxymethylcellulose, pectin, tragacanth, Veegum, acacia, sodium carboxymethylcellulose, hydroxypropyl methylcellulose, and polyvinylpyrrolidone. Preservatives include glycerin, methyl and propylparaben, benzoic acid, sodium benzoate and alcohol. Wetting agents include propylene glycol monostearate, sorbitan monooleate, diethylene glycol monolaurate, and polyoxyethylene lauryl ether. Solvents include glycerin, sorbitol, ethyl alcohol, and syrup. Examples of non-aqueous liquids utilized in emulsions include mineral oil and cottonseed oil. Organic acids include citric and tartaric acid. Sources of carbon dioxide include sodium bicarbonate and sodium carbonate.

[0061] It should be understood that many carriers and excipients may serve several functions, even within the same formulation.

[0062] In further embodiments, the pharmaceutical compositions provided herein may be provided as compressed tablets, tablet triturates, chewable lozenges, rapidly dissolving tablets, multiple compressed tablets, or enteric-coating tablets, sugar-coated, or film-coated tablets. Enteric-coated tablets are compressed tablets coated with substances that resist the action of stomach acid but dissolve or disintegrate in the intestine, thus protecting the active ingredients from the acidic environment of the stomach. Enteric-coatings include, but are not limited to, fatty acids, fats, phenylsalicylate, waxes, shellac, ammoniated shellac, and cellulose acetate phthalates. Sugar-coated tablets are compressed tablets surrounded by a sugar coating, which may be beneficial in covering up objectionable tastes or odors and in protecting the tablets from oxidation. Film-coated tablets are compressed tablets that are covered with a thin layer or film of
a water-soluble material. Film coatings include, but are not limited to, hydroxyethylcellulose, sodium carboxymethylcellulose, polyethylene glycol 4000, and cellulose acetate phthalate. Film coating imparts the same general characteristics as sugar coating. Multiple compressed tablets are compressed tablets made by more than one compression cycle, including layered tablets, and press-coated or dry-coated tablets.

[0063] The tablet dosage forms may be prepared from the active ingredient in powdered, crystalline, or granular forms, alone or in combination with one or more carriers or excipients described herein, including binders, disintegrants, controlled-release polymers, lubricants, diluents, and/or colorants. Flavoring and sweetening agents are especially useful in the formation of chewable tablets and lozenges.

[0064] The pharmaceutical compositions provided herein may be provided as soft or hard capsules, which can be made from gelatin, methylcellulose, starch, or calcium alginate. The hard gelatin capsule, also known as the dry-filled capsule (DFC), consists of two sections, one slipping over the other, thus completely enclosing the active ingredient. The soft elastic capsule (SEC) is a soft, globular shell, such as a gelatin shell, which is plasticized by the addition of glycerin, sorbitol, or a similar polyol. The soft gelatin shells may contain a preservative to prevent the growth of microorganisms. Suitable preservatives are those as described herein, including methyl- and propylparabens, and sorbic acid. The liquid, semisolid, and solid dosage forms provided herein may be encapsulated in a capsule. Suitable liquid and semisolid dosage forms include solutions and suspensions in propylene carbonate, vegetable oils, or triglycerides. Capsules containing such solutions can be prepared as described in U.S. Pat. Nos. 4,328,245; 4,409,239; and 4,410,545. The capsules may also be coated as known by those of skill in the art in order to modify or sustain dissolution of the active ingredient.

[0065] In other embodiments, the pharmaceutical compositions provided herein may be provided in liquid and semisolid dosage forms, including emulsions, solutions, suspensions, elixirs, and syrups. An emulsion is a two-phase system, in which one liquid is dispersed in the form of small globules throughout another liquid, which can be oil-in-water or water-in-oil. Emulsions may include a pharmaceutically acceptable non-aqueous liquids or solvent, emulsifying agent, and preservative. Suspensions may include a pharmaceutically acceptable suspending agent and preservative. Aqueous alcoholic solutions may include a pharmaceutically acceptable acetal, such as a di(lower alkyl) acetal of a lower alkyl aldehyde (the term "lower" means an alkyl having between 1 and 6 carbon atoms), e.g., acetaldehyde diethyl acetal; and a water-miscible solvent having one or more hydroxyl groups, such as propylene glycol and ethanol. Elixirs are clear, sweetened, and hydroalcoholic solutions. Syrups are concentrated
aqueous solutions of a sugar, for example, sucrose, and may also contain a preservative. For a
liquid dosage form, for example, a solution in a polyethylene glycol may be diluted with a
sufficient quantity of a pharmaceutically acceptable liquid carrier, e.g., water, to be measured
conveniently for administration.

[0066] Other useful liquid and semisolid dosage forms include, but are not limited to,
those containing the active ingredient(s) provided herein, and a dialkylated mono- or poly-
alkylene glycol, including, 1,2-dimethoxymethane, diglyme, triglyme, tetruglyme, polyethylene
glycol-350-dimethyl ether, polyethylene glycol-550-dimethyl ether, polyethylene glycol-750-
dimethyl ether, wherein 350, 550, and 750 refer to the approximate average molecular weight of
the polyethylene glycol. These formulations may further comprise one or more antioxidants,
such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate,
vitamin E, hydroquinone, hydroxycoumarins, ethanolamine, lecithin, cephalin, ascorbic acid,
malic acid, sorbitol, phosphoric acid, bisulfite, sodium metabisulfite, thiodipropionic acid and its
esters, and dithiocarbamates.

[0067] The pharmaceutical compositions provided herein for oral administration may
be also provided in the forms of liposomes, micelles, microspheres, or nanosystems. Micellar
dosage forms can be prepared as described in U.S. Pat. No. 6,350,458.

[0068] In other embodiments, the pharmaceutical compositions provided herein may be
provided as non-effervescent or effervescent, granules and powders, to be reconstituted into a
liquid dosage form. Pharmaceutically acceptable carriers and excipients used in the non-
effervescent granules or powders may include diluents, sweeteners, and wetting agents.
Pharmaceutically acceptable carriers and excipients used in the effervescent granules or powders
may include organic acids and a source of carbon dioxide.

[0069] Coloring and flavoring agents can be used in any of dosage forms according to
the present disclosure.

[0070] The pharmaceutical compositions provided herein may be formulated as
immediate or modified release dosage forms, including delayed-, sustained, pulsed-, controlled,
targeted-, and programmed-release forms.

[0071] In further embodiments, the pharmaceutical compositions provided herein may
be coformulated with other active ingredients which do not impair the desired therapeutic action,
or with substances that supplement the desired action.

EXAMPLES

[0072] A series of studies were undertaken to assess the anti-proliferative and anti-
survival effects on multiple myeloma cells by the combination of a therapeutically effective
amount of an anti-neoplastic alkylating agent, such as bendamustine, and a therapeutically
effective amount of a class I inhibitor of histone deacetylase, such as entinostat.

Materials and Methods
Reagents and antibodies

Bendamustine (Cephalon Inc., Frazer, PA) and entinostat (Syndax Pharmaceuticals, Inc., Waltham, MA) were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at 526 mmol/L and 20 mmol/L, respectively. The stock solutions were stored at -20°C.

The sources of antibodies for western blot were as follows: caspase-3 rabbit mAb (8G10), caspase-8 mouse mAb (1C12), PARP rabbit mAb, P-Histone H2A.X (Ser39) rabbit antibody, Histone H2A rabbit polyclonal antibody II, P-CHK1 (Ser345) (133D3) rabbit mAb, CHK1 rabbit antibody, P-CHK2 (Thr68) rabbit polyclonal antibody, and CHK2 rabbit polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA); β-actin mouse mAb (clone AC-75) (Sigma Chemical Co., St. Louis, MO). All other reagents were purchased from Sigma unless otherwise specified.

Cells and cell culture

Human MM cell line U266 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human MM cell line MM1.S and MM1.R [19] were kindly provided by Dr. Steven Rosen (Department of Medicine, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL). All cell lines were maintained in RPMI 640 cell culture medium supplemented with 10% fetal bovine serum (FBS) at a 37°C humidified atmosphere containing 95% air and 5% CO₂ and were split twice a week.

Cell proliferation assays

The CellTiter96™ AQ non-radioactive cell proliferation kit (Promega Corp., Madison, WI) was used to evaluate cell viability as we previously described [18]. In brief, cells were plated on 96-well plates with 0.1 ml complete medium containing 0.5% FBS as control, or 0.1 ml of the same medium with a series doses of bendamustine or entinostat alone or combination of them, and incubated for 72 h in a 37°C humidified atmosphere containing 95% air and 5% CO₂. After reading all wells at 490 nM with a 96-plate reader, the percentages of surviving cells from each group relative to control groups, defined as 100% survival, were determined by reduction of MTS.

Quantification of apoptosis
An apoptosis ELISA kit (Roche Diagnostics Corp., Indianapolis, IN) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously reported [18]. Western blot analysis

 massie Plus protein assay reagent (Pierce Chemical Co., Rockford, IL). Equal amounts of cell lysates were boiled in Laemmli SDS-sample buffer, resolved by SDS-PAGE, and western blot analysis with specific antibodies as provided in the descriptions of the figures.

2.6. Morphologic evaluation of mitotic catastrophe and apoptosis

 Cultured MM cells were harvested, resuspended with RPMI1640 medium, and cytocentrifuged for 1 minute at 1000 rpm. Cells were fixed in methanol for 5 minutes, and then stained in Jenner solution for 5 minutes. Samples were transferred into Giemsa solution for at least 45 minutes, and then rinsed in distilled water. Slides were examined under a photomicroscope (Olympus). Pathologists were blinded on each slide set regarding the treatment group. Cells that showed abnormal mitotic figures, chromatin condensation and fragmentation were counted against normal cells, and reported in percentage.

Statistical analysis

Statistical analyses of the experimental data were performed using a two-sided Student's t test. Significance was set at a P<0.05. Calculation of IC50, combination index (CI) and evaluation of synergy vs. antagonism between bendamustine and entinostat were performed using the Calcusyn software (Biosoft, Ferguson, MO), which was designed based on Chou-Talalay method [19; 20]. CI values less than, equal to and more than 1 represent synergistic, additive and antagonistic effects, respectively.

Exemplary Results

- Bendamustine in combination with entinostat enhances growth inhibition of MM cells, and is synergistic over a wide range of effects

To explore whether bendamustine or entinostat might have therapeutic potential against MM, cell growth assays were performed using U266, dexamethasone-sensitive (MM1.S) and dexamethasone-resistant (MM1.R) cell lines. Upon treatment with a serious dose of bendamustine or entinostat for 72 h, the proliferation of all three cell lines was significantly inhibited, although U266 cells were less sensitive to both agents than the other two cell lines.
(FIG. 1A and B). The response of MM cells to entinostat was in accordance with previous findings [18]. It appeared that MM1.R cells were more sensitive to the agents, especially entinostat, than MM1.S cells (Fig. 1A and B). Thus, both bendamustine and entinostat were able to inhibit proliferation of dexamethasone-sensitive and -resistant MM cells in a dose-dependent manner.

[0082] Next, it was assessed whether the combination of bendamustine and entinostat may further enhance their inhibitory effects on MM cells. After treating cells with single agent or their combinations in a fixed ratio for 72 h, a significant growth inhibition upon combinatorial treatment was observed as compared with either agent alone (FIG. 2A). The IC50s of bendamustine when used in combination with entinostat for U266, MM1.S and MM1.R cells were approximately 132.8, 13.7, and 34.5 μM/L, respectively. In contrast, The IC50s of bendamustine when used alone for U266, MM1.S and MM1.R cells were approximately 375, 86.9, and 83.8 μM/L, respectively. The combinatorial anti-proliferation activity was much more potent in MM1.S and MM1.R cells than that in U266 cells, which is consistent with single agent treatment. It should be emphasized that the combination enhanced inhibition dramatically at the concentration of 50 μM/L (bendamustine) and 0.2 μM/L (entinostat) in MM1.S cells, even though no inhibition was observed with entinostat (0.2 μM/L) alone (Fig. 2A). This result promoted us to further explore whether the two agents may have synergistic effect. A combination index (CI) analysis was performed according to the Chou-Talalay equation [19; 20]. The curves showed that bendamustine and entinostat exhibit a synergistic activity over a wide range of effects with CI = 0.53 ± 0.1339 at IC50s (fraction of cells affected = 0.5) in U266 cells. Similar results were obtained with MM1.S and MM1.R (FIG. 2B). In conclusion, the combination of bendamustine and entinostat synergistically induced growth inhibition in MM cells.

- Combination of bendamustine and entinostat significantly promotes MM cells undergoing apoptosis

[0083] To elucidate the molecular mechanism of bendamustine and entinostat-mediated anti-proliferation/anti-survival effects, it was assessed whether bendamustine and/or entinostat may induce apoptosis in MM cells. A specific apoptotic ELISA showed that entinostat alone (0.1 μM/L) induced minor apoptotic effect in U266, MM1.S and MM1.R cells (FIG. 3A). However, bendamustine alone induced apoptosis in a dose-dependent manner, and this effect was significantly enhanced after entinostat (0.1 μM/L) was added into bendamustine (FIG. 3A). Furthermore, western blot analysis revealed that the combination of bendamustine and entinostat as compared to either agent alone more potently induced PARP cleavage, the hall mark of
apoptosis, and activation of caspase-8 and -3 evidenced by the increases of cleaved caspase-8 and -3 in all three cell lines (FIG. 3B, C, D). These data indicate that entinostat significantly accelerates bendamustine-induced apoptosis in MM cells via caspase-dependent signaling pathways.

- Combination of bendamustine and entinostat significantly enhances DNA damage response associated with enhanced mitotic catastrophe

[0084] Since bendamustine induces DNA damage response and mitotic catastrophe of cancer cells, which are major mechanisms for treatment, an investigation was conducted to determine whether entinostat amplifies bendamustine-induced DNA damage response and mitotic catastrophe. The expression of DNA damage checkpoint proteins was examined after treating MM cells with single agent or their combinations for 24 h. Treatment with entinostat alone (0.1 μmol/L) did not increase P-H2A.X, P-CHK1, or P-CHK2 in either cell lines except MM1.S with minor increase of H2A.X phosphorylation (FIG. 4). However, the levels of P-H2A.X were dramatically increased following adding entinostat (0.1 μmol/L) to bendamustine treatment in all three cell lines, while the potent induction of P-CHK2 by combinatorial treatment was only observed in MM1.S cells (FIG. 4). In addition, bendamustine mainly upregulated P-CHK2 in U266 and MM1.S cells, whereas it enhanced CHK1 phosphorylation in all three cell lines. Moreover, morphologic observations revealed a significant increase in aberrant cells with mitotic catastrophe when treated with both bendamustine and entinostat as compared to either agent alone (FIG. 5), which was consistent with the results of our studies on apoptosis (FIG. 3) and DNA damage response (FIG. 4). Taken together, these data indicate that entinostat significantly enhances bendamustine-induced DNA damage response via induction of P-H2A.X and/or P-CHK2 in MM cells, and subsequently promotes the cells undergoing morphologic abnormalities and apoptosis.

Summary

Cell growth assays showed that bendamustine inhibited proliferation of MM cells in a dose-dependent manner. The IC50 of bendamustine for RPMI8226, U266 and MM1.S cells was approximately 662.6, 295.5, and 159.6 umol/L. Apoptotic-ELISA indicated that either bendamustine or entinostat was able to induce apoptosis in all three myeloma cells, and their combinations exhibited a much more potent activity to promote the MM cells undergoing apoptosis. Further studies with western blot analyses revealed that bendamustine in combination with entinostat dramatically induced PARP cleavage and activation of caspase-3 in all three MM cell lines, and also reduced the expression levels of Cyclin D1, phospho-Stat3 (P-stat3) and Stat3 in U266 cells. Moreover, studies on DNA damage response showed that phospho-histone
H2A.X, a hall marker of DNA double strand break, was significantly induced by the combinations of bendamustine and entinostat as compared to either agent alone in all three MM cell lines.

[0085] References:


Each of the foregoing references is incorporated herein by references for all purposes.
What is Claimed:

1. A method of treating multiple myeloma in a patient comprising administering to the patient a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof.

2. The method of claim 1 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is administered prior to the administration of the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

3. The method of claim 1 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is administered following the administration of the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

4. The method of claim 1 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is coadministered with the administration of the class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

5. The method of claim 1 wherein the anti-neoplastic alkylating agent is bendamustine or a pharmaceutically acceptable salt thereof.

6. The method according to claim 1 wherein the class I inhibitor of histone deacetylase is entinostat or a pharmaceutically acceptable salt thereof.

7. The method according to claim 1 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is administered in a ratio of from about 250:1 to about 2500:1 relative to said class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

8. A pharmaceutical dosage form comprising a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof.
9. The dosage form according to claim 8 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is administered in a ratio of from about 250:1 to about 2500:1 relative to said class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.

10. The dosage form according to claim 8 wherein the anti-neoplastic alkylating agent is bendamustine or a pharmaceutically acceptable salt thereof.

11. The dosage form according to claim 8 wherein the class I inhibitor of histone deacetylase is entinostat or a pharmaceutically acceptable salt thereof.

12. A kit comprising:

   at least one pharmaceutical dosage form comprising a therapeutically effective amount of an anti-neoplastic alkylating agent or a pharmaceutically acceptable salt thereof, and

   at least one pharmaceutical dosage form comprising a therapeutically effective amount of a class I inhibitor of histone deacetylase or a pharmaceutically acceptable salt thereof.

13. The kit according to claim 12 wherein the anti-neoplastic alkylating agent is bendamustine or a pharmaceutically acceptable salt thereof, and the class I inhibitor of histone deacetylase is entinostat or a pharmaceutically acceptable salt thereof.

14. The kit according to claim 12 wherein the anti-neoplastic alkylating agent or pharmaceutically acceptable salt thereof is provided in a ratio of from about 250:1 to about 2500:1 relative to said class I inhibitor of histone deacetylase or pharmaceutically acceptable salt thereof.
FIG. 1

A

B

U266

Survival (% Control)

Survival (% Control)

0 50 100 200 400
Benda (µmol/L)

0 0.5 1 2 4
Ent (µmol/L)

MM1.S

Survival (% Control)

Survival (% Control)

0 50 100 200 400
Benda (µmol/L)

0 0.25 0.5 1 2
Ent (µmol/L)
FIG. 2

Graph A shows the survival of U266 cells under different treatments with Benda, Ent, and Benda+Ent. The survival is expressed as a percentage of control and is plotted against the concentration of Benda (µmol/L) and Ent (µmol/L). Statistical significance is indicated by * (P<0.05) and ** (P<0.01).

Graph B illustrates the fractional effect against the combination index (CI) for the same treatments. The fractional effect is plotted against the concentration of Benda and Ent.
FIG. 4

A

U266

0 0 50 100 200 50 100 200 0 0 0 0 0 0 0 0 0.1 0.1 0.1

Benda (μmol/L) Ent (μmol/L)

P-H2AX H2AX P-CHK1 CHK1 P-CHK2 CHK2 β-actin

B

MM1.S

0 0 25 50 100 25 50 100 0 0 0 0 0 0 0 0 0.1 0.1 0.1

Benda (μmol/L) Ent (μmol/L)

P-H2AX H2AX P-CHK1 CHK1 P-CHK2 CHK2 β-actin

C

MM1.R

0 0 25 50 100 25 50 100 0 0 0 0 0 0 0 0 0.1 0.1 0.1

Benda (μmol/L) Ent (μmol/L)

P-H2AX H2AX P-CHK1 CHK1 P-CHK2 CHK2 β-actin
FIG. 5

A

Control

Ent (0.1 μmol/L)

Benda (100 μmol/L)

Ent + Benda

MM1.S

B

<table>
<thead>
<tr>
<th></th>
<th>U266</th>
<th>MM1.S</th>
<th>MM1.R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benda (μmol/L)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ent (μmol/L)</td>
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<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mitotic Catastrophe (%)</td>
<td>5.7</td>
<td>10.6</td>
<td>14.6</td>
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A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4184 A61K31/4406 A61K45/06 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.


Y 5, 10, 13

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

18 June 2013

Date of mailing of the international search report

27/06/2013

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
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Authorized officer
Schei, Rupert
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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