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(54) Title: CD80 ANTAGONISTS FOR TREATING NEOPLASTIC DISORDERS

(57) Abstract: Compositions comprising a heterocyclic compound and methods of using these compositions, alone or in combination with a second therapeutic agent, are provided for treating diseases or disorders in which CD80-expressing cells or regulatory T cell function contribute to or exacerbate the associated pathology. The disclosed combination therapies result in synergistic anti-tumor responses.

CD80 ANTAGONISTS FOR TREATING NEOPLASTIC DISORDERS

RELATED APPLICATIONS

Priority is claimed to US Application No. 11/539,153, filed 5 October 2006, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to the use of heterocyclic compounds for inhibition of CD80 signaling for treating cancers. The present invention also relates to the use of heterocyclic compounds in combination with other therapeutic agents, including for example, an anti-CD80 antibody, a B cell depleting antibody, and/or chemotherapy.

BACKGROUND OF THE INVENTION

The immune system possesses the ability to control the activation and inactivation of lymphocytes through various regulatory mechanisms during and after an immune response. Among these are mechanisms that specifically inhibit and/or turn off an immune response. When an antigen is presented by MHC molecules to a T cell receptor, the T cells become activated only in the presence of additional co-stimulatory signals. Thus, in the absence of accessory signals, there is no lymphocyte activation and either a state of functional inactivation termed anergy or tolerance is induced, or the T cell is specifically deleted by apoptosis. One such co-stimulatory signal involves the interaction of CD80 on specialized antigen-presenting cells with CD28 on T cells, which has been demonstrated to be essential for full T cell activation. (Lenschow *et al.*, *Annu. Rev. Immunol.* 14: 233-58 (1996)).

Much has been learned about the function of CD80 and their family members as costimulatory molecules for eliciting T cell responses (Freeman *et al.*, *J. Immunol.* 1043: 2714-22 (1989) and Hatchcock *et al.*, *J. Exp. Med.* 180(2): 631-40 (1999)). Various strategies, including antibody-based approaches aimed at blocking the interaction between CD80 and its ligand on T cells (CD28 CTLA-4), have been pursued as a treatment for autoimmune diseases and transplantation. (Anderson *et al.*, *Curr. Opn. Immunol.* 11(6): 677-83 (1999)). In addition to expression on activated B cells, CD80 is also expressed on malignant B cells. (Freeman *et al.*, *J. Immunol.* 1043: 2714-22 (1989) and Durfman *et al.*, *Blood* 90(11): 4297-4309 (1997)).

Various immunoregulatory antibodies, *i.e.*, antibodies that elicit a therapeutic benefit by modulating, enhancing or inhibiting a particular immune pathway, have been reported as anti-cancer agents. For example, such antibodies modulate the differentiation, proliferation, activation, and/or function of T cells and B cells, or other cells involved in regulation of humoral or cellular immunity through interaction with specific ligands. Such ligands include immune signaling molecules such as B7.1 (CD80), B7.2 (CD86), T cell regulatory molecules such as CD40L, CD40, and CD4. B cell depleting antibodies, *i.e.*, antibodies that specifically bind target antigens on B lymphocytes and which destroy or deplete B cells and/or interfere with B cell functions, are also useful anti-cancer agents. In some circumstances, these signaling molecules also function in non-malignant cells that support inflammatory and immune response within a tumor microenvironment. Thus, disruption of signaling within the tumor microenvironment offers another opportunity for therapeutic intervention.

To address the continuing need for effective cancer therapies, the present invention provides methods for treating CD80-positive malignancies using heterocyclic CD80 antagonists either alone or in combination with B cell depleting antibodies, immunoregulatory antibodies, or other chemotherapeutic agents. Additionally, the present invention provides methods of heterocyclic CD80 antagonists for modulating the function of non-malignant cells present in the tumor microenvironment to thereby disrupt conditions that support tumor growth and survival.

SUMMARY OF THE INVENTION

The present invention provides methods of treating a CD80-positive malignancy, including B cell malignancies (*i.e.*, lymphomas and leukemias) as well as solid tumors, using a compound of formula (I) as described herein. The compound of formula (I) can be used as a therapeutic agent or for selective targeting of a cytotoxic agent to CD80-positive malignant cells.

Also provided are combination therapies for treating CD80-positive malignancies. For example, a compound of formula (I) may be used in combination with one or more of a chemotherapeutic agent, a B cell depleting antibody, and an anti-CD80 antibody. The components of the disclosed combination therapies may be co-formulated or formulated separately for simultaneous or sequential administration. When used in combination, the compound of formula (I) and the chemotherapeutic agent, B cell depleting antibody, or anti-

CD80 antibody elicit a synergistic therapeutic effect (*e.g.*, an anti-cancer effect that is greater than the sum of effects elicited when either agent is used alone).

Further provided are methods of inducing apoptosis and/or cytotoxicity of CD80-positive malignant cells either *in vitro* or *in vivo*. According to such methods, CD80-positive malignant cells are contacted with a compound of formula (I), which is unconjugated or conjugated to a cytotoxin, whereby apoptosis or cytotoxicity is elicited. These methods may also include a step of contacting the cells with an additional agent having the desired effect, for example, chemotherapeutic agents, B cell depleting antibodies, and/or anti-CD80 antibodies.

Still further provided are methods of modulating non-malignant cells in a tumor microenvironment to thereby inhibit tumor progression. For example, the present invention provides a method of treating a subject having a malignancy, which comprises a tumor microenvironment of malignant and non-malignant cells, wherein regulatory T cell function contributes to or exacerbates the malignancy, comprising administering to the subject a therapeutically effective amount of a compound of formula (I). A compound of formula (I), as described herein, may also be used to suppress production of one or more inflammatory cytokines in a tumor microenvironment of a subject and/or to deplete non-malignant CD80-expressing cells in a tumor microenvironment of a subject.

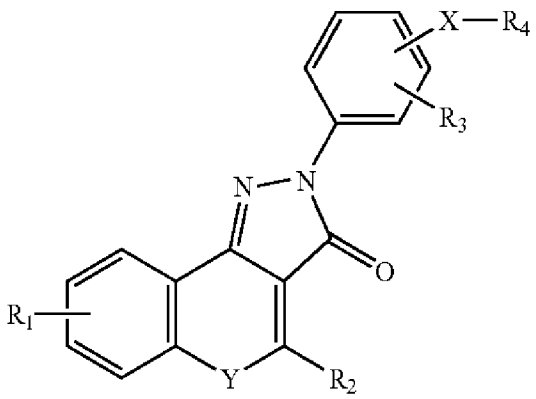
DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of treating CD80-positive malignancies in mammals by administration of quinoline derivatives alone or in combination with other therapeutic agents. In particular, disclosed heterocyclic compound of formula (I) may be administered in combination with other therapeutic agents, such as antibodies, chemotherapeutic agents, immunosuppressive agents, additional targeted anti-cancer drugs or systemic anti-cancer drugs, *etc.*, to thereby elicit synergistic or greater than additive therapeutic effects (*i.e.*, a therapeutic effect greater than either agent administered alone).

I. Heterocyclic CD80 Antagonists

The disclosed treatment methods employ heterocyclic compounds of formula (I) or a pharmaceutically or veterinarily acceptable salt thereof:

(I)



wherein

R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F;

R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl;

Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl;

X represents a bond or a divalent C_1 C_6 alkylene radical;

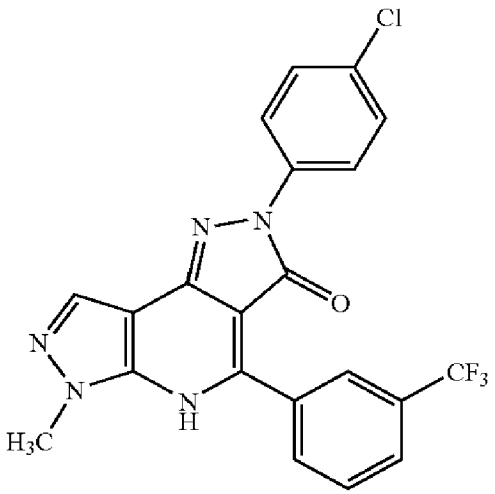
R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -$(Alk)_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and

Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7 cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and

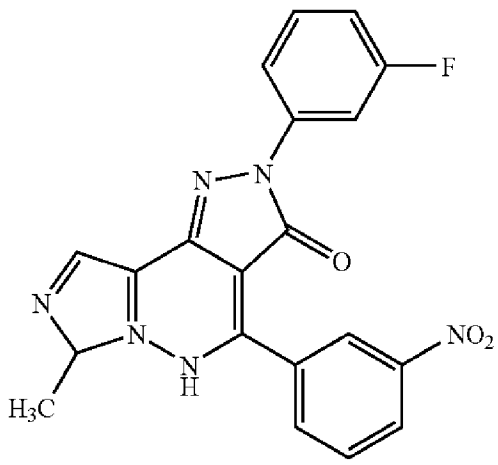
R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.

Representative molecules of formula (I) include fused pyrazolones having the following structures A and B and which inhibit CD80 signaling (Erbe *et al.*, *J. Biol. Chem.* 277(9): 7363-68 (2002)):

A



B



Heterocyclic compounds of formula (I) inhibit the interaction between CD80 and CD28 and thus the activation of T cells, thereby modulating immune responses. The compounds of formula (I) may exist in different tautomeric forms and all such forms may be used in the

methods of the present invention. Optical isomers and racemates of the compounds of general formula (I) may also be used.

Heterocyclic compounds of formula (I) are prepared essentially as described in U.S. Patent No. 7,081,456, the contents of which are incorporated by reference in their entirety herein. Salts of the heterocylic compounds may also be prepared, for example, physiologically acceptable acid addition salts such as hydrochlorides, hydrobromides, sulphates, methane sulphonates, p-toluenesulphonates, phosphates, acetates, citrates, succinates, lactates, tartrates, fumarates and maleates; and base addition salts, for example sodium, potassium, magnesium, and calcium salts. Where the compound contains an amino group, quaternary amino salts may also be prepared.

An alkylene molecule of formula (I) is a straight or branched alkyl chain having two unsatisfied valencies, for example —CH_2—, —CH_2CH_2—, —$CH_2CH_2CH_2$—, —$CH(CH_3)CH_2$—, —$CH(CH_2CH_3)CH_2CH_2CH_3$,and—$C(CH_3)_3$.

A heteroaryl moiety of formula (I) is a 5-or 6-membered aromatic ring containing one or more heteroatoms. Illustrative of such groups are thienyl, furyl, pyrrolyl, imidazolyl, benzimidazolyl, thiazolyl, pyrazolyl, isoxazolyl, isothiazolyl, triazolyl, thiadiazolyl, oxadiazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl.

Heterocyclyl or heterocyclic moieties of formula (I) include heteroaryl moieties as described above, and in particular means a 5-8 membered aromatic or non-aromatic heterocyclic ring containing one or more heteroatoms selected from S, N and O, including for example, pyrrolyl, furanyl, thienyl, piperidinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, pyrazolyl, pyridinyl, pyrrolidinyl, pyrimidinyl, morpholinyl, piperazinyl, indolyl, morpholinyl, benzofuranyl, pyranyl, isoxazolyl, quinuclidinyl, aza-bicyclo[3.2.1]octanyl, benzimidazolyl, methylenedioxyphenyl, maleimido and succinimido groups.

Substituted moieties of formula (I) include one or more of the following substituents, namely (C_1 C_6)alkyl, trifluoromethyl, (C_1 C_6)alkoxy (including the special case where a ring is substituted on adjacent ring C atoms by methylenedioxy or ethylenedioxy), trifluoromethoxy, (C_1 C_6)alkylthio, phenyl, benzyl, phenoxy, (C_3 C_8) cycloalkyl, hydroxy, mercapto, amino, fluoro, chloro, bromo, cyano, nitro, oxo, --COOH, —SO_2OH, —$CONH_2$, —SO_2NH_2, —COR^A, —$COOR^A$, —SO_2OR^A, —$NHCOR^A$, —$NHSO_2R^A$, —$CONHR^A$, —SO_2NHR^A, —NHR^A, —NR^AR^B, —$CONR^AR^B$ or —$SO_2NR^AR^B$ wherein R^A and R^B are independently a (C_1 C_6)alkyl

group. In the case where a substituted moiety includes a (C_3 C_8)cycloalkyl, phenyl, benzyl or phenoxy, the ring thereof may itself be substituted with any of the foregoing, except (C_3 C_8)cycloalkyl phenyl, benzyl, or phenoxy.

Carbocyclyl or carbocyclic moieties of formula (I) include 5-8 membered rings whose ring atoms are all carbon.

Some compounds of formula (I) contain one or more chiral centres because of the presence of asymmetric carbon atoms. The presence of asymmetric carbon atoms gives rise to stereoisomers or diastereoisomers with R or S stereochemistry at each chiral centre. CD80 antagonists of formula (I) may include such stereoisomers and diastereoisomers and mixtures thereof.

Heterocyclic CD80 antagonists of the invention have anti-cancer activity, such as induction of apoptosis (*e.g.*, as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles or apoptotic bodies), induction of cytotoxicity (cell lysis or other cell killing), depletion of malignant cells, and/or enhancement of anti-cancer activity of a known anti-cancer agent (*e.g.*, enhancement of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) of a therapeutic antibody). Anti-cancer activities also include inhibition or delay of tumor growth. Representative techniques for demonstrating anti-cancer activities of a CD80 antagonist of the invention are described in Examples 1-3 and 5-8.

As described herein, a compound of formula (I) includes molecules that block CD80/CD28 signaling to thereby attenuate the immunosuppressive effects of regulatory T cells and Th2 helper T cells, which then permits tumor-reactive T cells to mediate anti-tumor activity. CD80-CD28 signaling is a major coregulatory pathway for regulatory T cell and Th2 helper cell differentiation and maintenance, and T cells dramatically decrease in the absence of CD80 costimulation. *See* Zheng *et al.*, *J. Immunol.*, 172:2778-2784 (2004); Liang *et al.*, *J. Exp. Med.* 201: 127-137 (2005); and Tang *et al.*, *J. Immunol.*, 171:3348-3352 (2003). Selective blockade of CD80/CD28 signaling, *i.e.*, in the absence of blockade of CD80/CTLA4 signaling, may offer additional therapeutic efficacy given that the negative modulation of immune responses by CD80/CTLA4 signaling is not disrupted. CD80 antagonists having selective blockade of CD80/CD28 signaling are readily characterized as such using known techniques, for example as described in Example 4.

II. Conjugates and Other Modifications of a CD80 Antagonist

Heterocyclic compounds of formula (I) may also be used as targeting molecules to direct a cytotoxic agent to CD80 expressing malignant cells. Thus, the CD80 antagonists used in the methods disclosed are optionally conjugated to a cytotoxic agent, *i.e.*, a substance that inhibits or prevents normal cellular functions and/or causes destruction of cells. Cytotoxic agents can be covalently bound to the formula (I) CD80 antagonist by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents. The linker may be a cleavable linker facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Charm *et al.*, *Cancer Research* 52:127-131 (1992)) may be used.

Representative cytotoxins include radioactive isotopes, chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof. Cytotoxic agents may be associated or bound to heterocyclic compounds of formula (I) either directly or indirectly, for example, using stable or labile linkers or by using biological or synthetic matrices. A formula (I) CD80 antagonist may also be conjugated to a first member of a binding pair (*e.g.*, streptavidin) for utilization in tumor pretargeting. According to this approach, the formula (I)/binding partner conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent, and then administration of a second member of the binding pair (*e.g.*, avidin) which is conjugated to a cytotoxic agent as described herein.

A chemotherapeutic agent is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamime nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin,

carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.,* paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, NJ) and doxetaxel (Taxotere, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and antiandrogens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Cytotoxic agents that may be used to prepare formula (I) conjugates include, cytostatic agents, alkylating agents, antimetabolites, anti-proliferative agents, tubulin binding agents, hormones and hormone antagonists, and the like. Exemplary cytostatics that are compatible with the present invention include CHOPP (cyclophosphamide, doxorubicin, vincristine, prednisone and procarbazine); CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone); COP (cyclophosphamide, vincristine, prednisone); CAP-BOP (cyclophosphamide, doxorubicin, procarbazine, bleomycin, vincristine and prednisone); m-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone, and leucovorin; ProMACE-MOPP (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leukovorin, mechloethamine, vincristine, prednisone and procarbazine); ProMACE-CytaBOM (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leukovorin, cytarabine, bleomycin and vincristine); MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin and leukovorin); MOPP (mechloethamine, vincristine, prednisone and procarbazine); ABVD (adriamycin/doxorubicin, bleomycin, vinblastine and dacarbazine); MOPP (mechloethamine, vincristine, prednisone and procarbazine) alternating with ABV (adriamycin/doxorubicin, bleomycin, vinblastine); MOPP (mechloethamine, vincristine, prednisone and procarbazin) alternating with ABVD(adriamycin/doxorubicin, bleomycin, vinblastine and dacarbazine); ChlVPP (chlorambucil, vinblastine, procarbazine, prednisone); IMVP-16 (ifosfamide, methotrexate, etoposide); MIME (methyl-gag, ifosfamide, methotrexate, etoposide); DHAP (dexamethasone, high-dose cytaribine and cisplatin); ESHAP (etoposide, methylpredisolone, HD cytarabine, and cisplatin); CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone and bleomycin); CAMP (lomustine, mitoxantrone, cytarabine and prednisone); and CVP-1 (cyclophosphamide, vincristine and prednisone); DHAP (cisplatin, high-dose cytarabine and dexamethasone); CAP (cyclophosphamide, doxorubicin, cisplatin); PV (cisplatin, vinblastine or vindesine); CE (carboplatin, etoposide); EP (etoposide, cisplatin); MVP (mitomycin, vinblastine or vindesine, cisplatin); PFL (cisplatin, 5-flurouracil, leucovorin); IM (ifosfamide, mitomycin); IE (ifosfamide, etoposide); IP (ifosfamide, cisplatin); MIP (mitomycin, ifosfamide, cisplatin); ICE (ifosfamide, carboplatin, etoposide); PIE (cisplatin, ifosfamide, etoposide); Viorelbine and cisplatin; Carboplatin and paclitaxel; CAV (cyclophosphamide, doxorubicin, vincristine); CAE (cyclophosphamide, doxorubicin, etoposide); CAVE

(cyclophosphamide, doxorubicin, vincristine, etoposide); EP (etoposide, cisplatin); and CMCcV (cyclophosphamide, methotrexate, lomustine, vincristine).

Additional representative cytotoxins include members or derivatives of the enediyne family of anti-tumor antibiotics, including calicheamicin, esperamicins or dynemicins. These toxins are extremely potent and act by cleaving nuclear DNA, leading to cell death. Unlike protein toxins which can be cleaved *in vivo* to give many inactive but immunogenic polypeptide fragments, toxins such as calicheamicin, esperamicins and other enediynes are small molecules which are essentially non-immunogenic. These non-peptide toxins are chemically-linked to the dimers or tetramers by techniques which have been previously used to label monoclonal antibodies and other molecules. These linking technologies include site-specific linkage via the N-linked sugar residues present only on the Fc portion of the constructs. Such site-directed linking methods have the advantage of reducing the possible effects of linkage on the binding properties of the constructs.

For example, conjugates of a heterocyclic compound of formula (I) and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Patent No. 5,208,020), a trichothene, and CC1065, may be used in the disclosed therapeutic methods. In one such example, the CD80 antagonist is conjugated to one or more maytansine molecules (*e.g.*, about 1 to about 10 maytansine molecules per B-cell antagonist). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified CD80 antagonists (Chari *et al.*, *Cancer Research 52*: 127-131 (1992)) to generate a maytansinoid-CD80 antagonist conjugate.

As another example, the heterocyclic compound of formula (I) is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and Φ_1^I (Hinman *et al.*, *Cancer Research 53*: 3336-3342 (1993) and Lode *et al.*, *Cancer Research 58*: 2925- 2928 (1998)).

Cytokines are proteins released by one cell population which act on another cell as intercellular mediators. Examples of cytokines include lymphokines, monokines, and traditional polypeptide hormones. Also included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone;

parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-13; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocytemacrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-la, IL-2, IL-g, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-15; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

A prodrug is a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. *See e.g.,* Wilman, "Prodrugs in Cancer Chemotherapy," *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986); Stella *et al.,* "Prodrugs: A Chemical Approach to Targeted Drug Delivery," in Borchardt *et al.,* (ed.), Directed Drug Delivery, pp. 247-267, Humana Press (1985). Prodrugs which may be used to prepare formula (I) conjugates include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, 13-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, any of the chemotherapeutic agents described herein above.

The CD80 antagonists of the present invention may also be conjugated with a prodrug activating enzyme which converts a prodrug (*e.g.,* a peptidyl chemotherapeutic agent, *see* PCT International Publication No.W081/01145) to an active anti-cancer drug. *See*, for example, PCT

International Publication No. WO 88/07378 and U.S. Patent No. 4,975,278. The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form. Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic5-fluorocytosine into the anti-cancer drug, fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate cleaving enzymes such as 13-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; 13-lactamase useful for converting drugs derivatized with 13-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as abzymes, can be used to convert the prodrugs of the invention into free active drugs (*see e.g.,* Massey, *Nature* 328:457-458 (1987)). Antagonist-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

Immunosuppressive agents are substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (*see* U.S. Patent. No. 4,665,077, the disclosure of which is incorporated herein by reference), azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Patent. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.,* prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor antagonists including anti-interferon-α, β- or δ-antibodies, anti-tumor necrosis factor-α antibodies, anti-tumor necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD 1 la and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous

anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (PCT International Publication No. WO 90/08187), streptolanase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen *et al.,* U.S. Patent No. 5,114,721); T cell receptor fragments (Offner *et al., Science,* 251: 430-432 (1991); PCT International Publication No. WO 90/11294; Laneway, *Nature,* 341: 482 (1989); and PCT International Publication No. WO 91/01133); and T cell receptor antibodies such as T10B9 (European Patent No. 340,109).

A variety of radioactive isotopes are available for the production of radioconjugated antagonists, including α-emitters, β-emitters, and auger electrons. Particular examples include ^{18}fluorine, ^{64}copper, ^{65}copper, ^{67}gallium, ^{68}gallium, ^{77}bromine, ^{80m}bromine, ^{95}ruthenium, ^{97}ruthenium, ^{103}ruthenium, ^{105}ruthenium, ^{99m}technetium, ^{107}mercury, ^{203}mercury, ^{123}iodine, ^{124}iodine, ^{125}iodine, ^{126}iodine, ^{131}iodine, ^{133}iodine, ^{111}indium, ^{113}indium, ^{99m}rhenium, ^{105}rhenium, ^{101}rhenium, ^{186}rhenium, ^{188}rhenium, ^{121}mtellurium, ^{99}technetium, ^{122m}tellurium, ^{125m}tellurium, ^{165}thulium, ^{167}thulium, ^{168}thulium, ^{90}yttrium, ^{213}bismuth, ^{213}lead, and ^{225}actinium, and nitride or oxide forms derived there from.

Enzymatically active toxins and fragments thereof which can be used to prepare formula (I) conjugates include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa),* ricin A chain, abrin A chain, modeccin A chain, alpha sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. *See*, for example, PCT International Publication No. WO 93/21232. Compounds with nucleolytic activity *(e.g.,* a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase) may also be used.

A formula (I) CD80 antagonist may be linked to one of a variety of nonproteinaceous polymers, *e.g.,* polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol, for example, to improve solubility, reduce toxicity, and/or extend circulating half-life.

III. Therapeutic Applications

CD80-positive malignancies treatable using the disclosed methods include include hematologic malignancies such as lymphomas and leukemias as well as solid tumors. Representative hematologic malignancies that are amenable to treatment include leukemias, such as ALL-L3 (Burkitt's type leukemia), chronic lymphocytic leukemia (CLL) and monocytic cell leukemias. The methods of the present invention are also effective in treating a variety of B-cell lymphomas, including Hodgkin's lymphoma and non-Hodgkin's lymphoma, such as low grade/follicular non-Hodgkin's lymphoma (NHL), cell lymphoma (FCC), mantle cell lymphoma (MCL), diffuse large cell lymphoma (DLCL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, and Waldenstrom's Macroglobulinemia. Representative solid tumors that are amenable to treatment according to the disclosed invention include primary and metastatic tumors in breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder, bile ducts, small intestine, urinary tract including kidney, bladder and urothelium, female genital tract, cervix, uterus, ovaries, male genital tract, prostate, seminal vesicles, testes, an endocrine gland, thyroid gland, adrenal gland, pituitary gland, skin, bone, soft tissues, blood vessels, brain, nerves, eyes, meninges.

For the treatment of malignant diseases, therapeutic or anti-cancer effects may be measured using clinical outcomes such as reduction in tumor mass and/or the number of nodules related to a hematologic malignancy, reduction of abnormally large spleen or liver, reduction or disappearance of metastases, and progression-free survival. Additional indices of therapeutic effect include reduction of the number of malignant cells, or reduced or slowed growth of malignant cells. The change is assessed relative to a control level or sample, for example a level of a therapeutic index observed in a subject prior to administration of a CD80-targeted therapeutic. For example, a change in any of the above-noted indices may be a change of at least about two-fold greater or less than a control level, or at least about at least about five-fold greater or less than a control level, or at least about ten-fold greater or less than a control level, at least about twenty-fold greater or less than a control level, at least about fifty-fold greater or less than a control level, or at least about one hundred-fold greater or less than a control level. A change in the above-noted indices may also be observed as a change of at least 20% compared to a

control level, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 100%, or more.

The present invention also provides methods for modulation of non-malignant cells within the tumor microenvironment, which normally function to regulate inflammation as well as innate and adaptive immunity, and which directly or indirectly support tumor progression, by administration of quinoline derivatives that function as CD80 antagonists. In this aspect of the invention, non-malignant cells of a tumor microenvironment are modulated such that the contribution of those cells to tumor progression is inhibited, and the function of those cells in supporting regulatory T cell function is also inhibited. For example, methods are provided for using a compound of formula (I) for enhancing cancer immunity or reducing immune or inflammatory support of tumor progression in a subject by modulating immune cells present in the tumor microenvironment, for example, by decreasing the number of immunoregulatory or inflammatory cells such as antigen presenting cells (*e.g.*, macrophages, dendritic cells, B cells) or myeloid-derived suppressor cells (*e.g.*, myeloid-derived monocytes and tie-2-expressing monocytes) present within the tumor microenvironment, inhibiting T cell subsets that function to support tumor progression (*e.g.*, regulatory T cell and Th2 helper T cells), and/or suppressing production of one or more inflammatory cytokines in a tumor microenvironment. *See e.g.*, U.S. Provisional Application No. 60/908,645, filed March 28, 2007, which is incorporated by reference herein in its entirety.

Cells of a tumor microenvironment comprise malignant cells in association with non-malignant cells that support their growth and survival. The non-malignant cells, also called stromal cells, occupy or accumulate in the same cellular space as malignant cells, or the cellular space adjacent or proximal to malignant cells, which modulate tumor cell growth or survival. Non-malignant cells of the tumor microenvironment include fibroblasts, myofibroblasts, glial cells, epithelial cells, adipocytes, vascular cells (including blood and lymphatic vascular endothelial cells and pericytes), resident and/or recruited inflammatory and immune (*e.g.*, macrophages, dendritic cells, myeloid suppressor cells, granulocytes, lymphocytes, etc.), resident and/or recruited stem cells that are capable of giving rise to or differentiating into any of the above-noted non-malignant cells, and any functionally distinct subtypes of the above-noted cells as known in the art. These cells actively participate in tumor cell growth and metastasis.

In performing the methods disclosed herein, a tumor microenvironment is identified using one or more of the following criteria: (a) a region comprising non-malignant cells which share the same physiological environment, or which are directly adjacent to malignant cells; (b) the extended tumor region; (c) an area of inflammation surrounding or proximal to a tumor; (d) an area in which the number or rate of proliferation of regulatory T cells is elevated; and (e) an area in which macrophages, dendritic cells, or myeloid-derived suppressor cells are elevated. Within the context of non-solid tumor types, the tumor microenvironment may also be determined by the local cell-cell interactions between malignant cells and between malignant cells and any adjacent or nearby non-malignant cells. Such interactions may include, for example, cell adhesion events and/pr paracrine effects of soluble mediators produced by one cell (malignant or non-malignant) on another cell (malignant or non-malignant) in the tumor microenvironment.

Among the non-malignant cells of a tumor microenvironment are regulatory T cells, which are observed in higher frequencies in a number of tumors, including Hodgkin's lymphoma, non-Hodgkin's lymphoma (Shi *et al.*, *Ai Zheng*. 23(5):597-601 (2004) (abstract only)), malignant melanoma (Viguier *et al.*, *J. Immunol*. 173(2):1444-53 (2004); Javia *et al.*, *J. Immunother*. 26(1):85-93 (2003), and cancers of the ovary (Woo *et al.*, *Cancer Res*. 61(12):4766-72 (2001), gastrointestinal tract (Ichihara *et al.*, *Clin. Cancer Res*. 9(12):4404-4408 (2003); Sasada *et al.*, *Cancer* 98(5):1089-1099 (2003), breast (Liyanage *et al.*, *J. Immunol*. 169(5):2756-2761 (2002), lung (Woo *et al.*, *Cancer Res*. 2001, 61(12):4766-72 (2001), and pancreas (Liyanage *et al.*, *J. Immunol*. 169(5):2756-2761 (2002)). The regulatory T cells are recruited to the tumor site in response to chemokines secreted by the tumor cells. *See e.g.*, Curiel *et al.*, *Nat. Med*. 10:942-949 (2004). An increase in the number of regulatory T cells may also correlate with poor prognosis (Curiel *et al.*, *Nat. Med*. 10:942-949 (2004); Sasada *et al.*, *Cancer* 98:1089-1099 (2003)). Conversely, regulatory T cells are observed to decrease following chemotherapy (Beyer et al., *Blood* 106:2018-2025 (2005)).

Regulatory T cells may be identified as enriched within the $CD4^{+}CD25^{hi}$ population and may be further characterized by expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), CC chemokine receptor 4 (CCR4), forkhead box p3 (FOXP3), CC chemokine receptor 6 (CCR6), and/or CD30. In addition, regulatory T cells may be $CD62L^{hi}$, $CD45RB^{lo}$, $CD45RO^{hi}$, and/or

$CD45RA^-$. *See* McHugh *et al.*, *J. Allergy Clin. Immunol.* 110:693-701 (2002); Hori *et al.*, *Science* 299:1057-1061 (2003); Iellem *et al.*, *J. Exp. Med.* 194:847-853 (2001); U.S. Patent Application Publication No. 2006/0063256.

Helper T cells include $CD4^+CD25^-$ Th1 cells and Th2 cells. Th1 cells promote cellular immunity, *i.e.*, the induction of antigen-specific $CD8^+$ cytotoxic T cells. Th2 helper T cells can suppress Th1 cell-mediated cellular immunity, for example, by secreting cytokines that inhibit interferon-γ production by Th1 cells (Fiorentino et al., *J. Exp., Med.* 170: 2081-2095 (1989)).

The activation of regulatory T cells and Th2 helper T cells creates an inflammatory tumor microenvironment that attracts macrophages. The macrophages promote survival of malignant cells by secreting cytokines such as IL-1 and IL-6. Macrophages also secrete chemokines for the recruitment of additional immune effector cells to the tumor microenvironment, for example, IL-8, which attracts neutorphils, and MIP-1α, which attracts leukocytes. Macrophages further secrete angiogenic factors, which contribute to vascularization of the tumor.

When assessing a level of any one of the above-noted qualities of a tumor microenvironment, *e.g.*, a level of inflammation or a number of regulatory T cells, the level is assessed relative to a reasonable control level. For example, a relevant control may comprise a sample taken from a tumor-bearing subject and from a same tissue and analogous region on the contralateral side of the subject. As another control, a sample may be taken from a same tissue and analogous region from a similarly situated (age, gender, overall health, *etc.*) subject who lacks a tumor. In the case of assessment of treatment-dependent response, post-treatment effects may also be ascertained through parallel analysis of a pre-treatment control sample.

Thus, a therapeutic or anti-cancer effect of a CD80-targeted therapeutic may also be assessed as a reduction of regulatory T cells and/or Th2 helper cells in the tumor microenvironment, inhibition of regulatory T cell and/or Th2 helper cell activation in the tumor microenvironment, or inhibition of regulatory T cell function to thereby produce an anti-cancer effect. For example, inhibition of regulatory T cell function may be observed as reduced production or secretion of pro-inflammatory cytokines (*e.g.*, interleukin-2, interleukin-4, interleukin-10, interleukin-12, transforming growth factor beta (TGF-β), and interferon-γ (IFN-γ), dis-inhibition of Th1 help for cell-mediated immunity; dis-inhibition of Th2 help for antibody production; and dis-inhibition of CD8+ cytotoxic T lymphocytes. Additional relevant changes in the tumor microenvironment indicative of anti-cancer activity include potentiation of

natural killer cell activity, a decrease in vascularization, and depletion of antigen presenting cells such as macrophages or dendritic cells, or depletion of other CD80-expressing cells, such as myeloid-derived suppressor cells. Still additional indicators of anti-tumor efficacy include a shift in gene expression to Immune Response 1 and Immune Response 2 signature gene expression profiles by non-malignant cells, as described by Dave et al., *N. Engl. J. Med.*, 351(21):2159-2169 (2004).

When quantifying a level of any of the above-described criteria for defining a tumor microenvironment or a therapeutic effect based upon a change in a tumor microenvironment, a difference when assessed relative to a control level is identified as a difference of at least about two-fold greater or less than a control level, or at least about at least about five-fold greater or less than a control level, or at least about ten-fold greater or less than a control level, at least about twenty-fold greater or less than a control level, at least about fifty-fold greater or less than a control level, or at least about one hundred-fold greater or less than a control level. A difference in the above-noted criteria when assessed relative to a control level may also be observed as a difference of at least 20% compared to a control level, such as at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 100%, or more. For example, an elevated number of regulatory T cells may be assessed relative to a known physiological level of regulatory T cells in a patient lacking a tumor. Based upon the foregoing, one of ordinary skill in the art (*e.g.*, a clinician in oncology) would be able to readily identify a region constituting a tumor microenvironment in a patient using the above-noted criteria and selection of appropriate controls. A subject may also have one or more tumor microenvironments, each associated with a distinguishable tumor. The characteristics of a tumor microenvironment may vary in relation to the tissue site of the tumor, the grade of a tumor, the stage of a tumor, the morphological or molecular phenotype of a tumor, *etc.* A tumor microenvironment may also change as a tumor progresses.

IV. Combination Therapies

The disclosed heterocyclic compounds of formula (I) may be administered as an initial treatment, or for treatment of conditions that are unresponsive to conventional therapies. Thus, the disclosed methods may be used for treating patients that have relapsed or are refractory to prior treatments using a same or different therapeutic agent or combination of agents. In

addition, the disclosed heterocyclic compound of formula (I) may be used in combination with other therapeutic agents (*e.g.*, antibodies, chemotherapeutic agents, *etc.*), or other therapies (*e.g.*, surgical excision, radiation, *etc.*) to thereby elicit synergistic or greater than additive therapeutic effects and/or to reduce hepatocytotoxicity of some therapeutic agents. A compound of formula (I) may be co-administered or co-formulated with additional agents, or formulated for consecutive administration in either order. Measurable therapeutic effects are described herein above. For example, a synergistic therapeutic effect may be an effect of at least about two-fold greater than the therapeutic effect elicited by a single agent, or at least about at least about five-fold greater, or at least about ten-fold greater, or at least about twenty-fold greater, or at least about fifty-fold greater, or at least about one hundred-fold greater.

Representative agents useful for combination therapy include any of the drugs useful for preparation of compound of formula (I)/drug conjugates described herein above. Additional representative agents include cytotoxins, radioisotopes, chemotherapeutic agents, immunomodulatory or immunoregulatory agents, anti-angiogenic agents, anti-proliferative agents, pro-apoptotic agents, cytostatic and cytolytic enzymes (*e.g.*, RNAses), enzyme inhibitors (*e.g.*, proteasome inhibitors), therapeutic antibodies, antibody/drug conjugates and tumor vaccines. Additional agents include a therapeutic nucleic acid, such as a gene encoding an immunomodulatory agent, an anti-angiogenic agent, an anti-proliferative agent, or a pro-apoptotic agent. These drug descriptors are not mutually exclusive, and thus a therapeutic agent may be described using one or more of the above-noted terms.

Additional agents that may be used in combination with CD80 antagonists include agents that block interaction of B lymphocyte stimulator (BLyS) with one or more of its receptors, B cell activating factor receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), or B-cell maturation antibody (BCMA). For example, useful agents include antibodies that specifically bind the BLyS ligand or antibodies that specifically bind one or more of its receptors, including any of the antibody types described herein. Small molecule inhibitors of the interaction of BLyS with one or more of its receptors may also be used.

Heterocyclic compounds of formula (I) of the invention may also be used in combination with other therapeutic antibodies, including immunoregulatory antibodies and B cell depleting antibodies, and antibody/drug conjugates. Representative antibodies and antibody/drug

conjugates include CD19, anti-CD20 (*e.g.*, RITUXAN®, ZEVALIN®, BEXXAR®), anti-CD22 antibodies, anti-CD33 antibodies (*e.g.*, MYLOTARG®), anti-CD33 antibody/drug conjugates, anti-Lewis Y antibodies (*e.g.*, Hu3S193, Mthu3S193, AGmthu3S193), anti-HER-2 antibodies (*e.g.*, HERCEPTIN® (trastuzumab), MDX-210, OMNITARG® (pertuzumab, rhuMAb 2C4)), anti-CD52 antibodies (*e.g.*, CAMPATH®), anti-EGFR antibodies (*e.g.*, ERBITUX® (cetuximab), ABX-EGF (panitumumab)), anti-VEGF antibodies (*e.g.*, AVASTIN® (bevacizumab)), anti-DNA/histone complex antibodies (*e.g.*, ch-TNT-1/b), anti-CEA antibodies (*e.g.*, CEA-Cide, YMB-1003) hLM609, anti-CD47 antibodies (*e.g.*, 6H9), anti-VEGFR2 (or kinase insert domain-containing receptor, KDR) antibodies (*e.g.*, IMC-1C11), anti-Ep-CAM antibodies (*e.g.*, ING-1), anti-FAP antibodies (*e.g.*, sibrotuzumab), anti-DR4 antibodies (*e.g.*, TRAIL-R), anti-progesterone receptor antibodies (*e.g.*, 2C5), anti-CA19.9 antibodies (*e.g.*, GIVAREX®) and anti-fibrin antibodies (*e.g.*, MH-1). The compound of formula (I) may also be used in combination with systemic anti-cancer drugs, such as epithilones (BMS-247550, Epo-906), reformulations of taxanes (ABRAXANE®, XYOTAX®), microtubulin inhibitors (MST-997, TTI-237), or with targeted cytotoxins such as CMD-193 and SGN-15. Additional useful anti-cancer agents include TAXOTERE®, TARCEVA®, GEMZAR® (gemcitabine), 5-FU, AVASTIN®, ERBITUX®, TROVAX®, anatumomab mafenatox, letrazole, docetaxel, and anthracyclines. In addition, antibodies or antibody fragments having substantial similar activities of the above-noted antibodies, or variants of the above-noted antibodies, may be prepared as described further below.

For example, a compound of formula (I) may be administered in combination with an anti-CD80 antibody and/or antiCD20 antibody. Another example comprises the administration of a compound of formula (I) in combination with one or more therapeutic agents, such as chemotherapeutic agents, cytotoxins, or immunosuppressive agents. The administration or application of the various components of the combined therapeutic regimen may be timed to enhance the overall effectiveness of the treatment. A skilled artisan (*e.g.,* an oncologist) would be readily be able to discern effective combined therapeutic regimens without undue experimentation.

For the treatment of Hodgkin's Disease, CD80-targeted therapeutics of the invention may be used in combination with antibodies that bind to antigens expressed on Hodgkin Reed Sternberg (HRS) cells or cells of the infiltrate surrounding HRS cells. Such antigens that are

useful for targeting of HRS cells include CD30, CD40, RANK, TRAIL, Notch, LMP, IL-13, CD20, CD52, and CCR4. Other useful agents for combination therapies for treating Hodgkin's Diseae include proteosome inhibitors (*e.g.*, Bortezomib (PS-341; Millennium Pharmaceuticals) and MG-132 (Tokyo, Metroplitan Institute of Medical Science)), histone deacytylase inhibitors (*e.g.*, depsipeptide (FK228; Gloucester Pharmaceuticals) and suberoylanilide hydroxamic acid (SAHA; Aton Pharma)), and small molecules and peptides that may be used to induce apoptosis of HRS cells, including triterpenoids, such as CDD (RTA401, Reata Discovery) and 17-allylamino-17-demethoxy-gledanamycin (*see e.g.,* Kamal et al., *Trends. Mol. Med.* 10, 283-290 (2004)), N-acetyl-leucinyl- leucynil-norleucynal, N-acetyl-leucinyl leucynil-methional, carbobenzoxyl-leucinyl-leucynil-norvalinal, carbobenzoxyl-leucinyl-leucynil-leucynal, β-lactone, bactacystine, boronic acid peptides, ubiquitin ligase inhibitors, cyclosporin A, and deoxyspergualin.

For modulation of a tumor micorenvironemnt, CD80-targeted therapeutics of the invention may also be used in combination with agents that promote anti-tumor immunity, for example, tumor vaccines. Many such vaccines are known in the art, including vaccines that incorporate tumor-specific cytotoxic as well as helper epitopes. Additional agents may prevent the induction of $CD8^+$ cytolytic T lymphocyte anergy. Still further, CD80-targeted therapeutics of the invention may be used in combination with agents that enhance immune activity, for example, anti-CTLA4 antibodies, other agents that block CTLA4, anti-PD1 antibodies, and additional agents that release inhibitory controls on T cell activation and proliferation.

IV.A. Therapeutic Antibodies Used In Combination With A CD80 Antagonist

The term antibody refers to an immunoglobulin protein, or antibody fragments that comprise an antigen binding site (*e.g.,* Fab, modified Fab, Fab', $F(ab')_2$ or Fv fragments, or a protein having at least one immunoglobulin light chain variable region or at least one immunoglobulin heavy chain region). Antibodies of the invention include chimeric antibodies, humanized antibodies, human antibodies, diabodies, tetrameric antibodies, single chain antibodies, tetravalent antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), domain-specific antibodies that recognize a particular epitope (*e.g.*, antibodies that recognize an epitope bound by the anti-CD80 or anti-CD20 antibodies.

Native antibodies are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term variable refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a 13-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the B -sheet structure.

The term hypervariable region refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a complementarity determining region or CDR (*e.g.,* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.,* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)), and/or those residues from a hypervariable loop (*e.g.,* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia *et al., J. Mol. Biol.* 196:901-917 (1987)). Framework or FR residues are those variable domain residues other than the hypervariable region residues as herein defined.

The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.,* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.,* IgGI, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called alpha, delta, epsilon, gamma and mu, respectively. The heavy-chain constant domains will complete the gamma-1, gamma-2, gamma-3 and gamma-4 constant region. These constant domains may also comprise modifications to enhance antibody stability such as the P and E modification disclosed in U.S. Patent No. 6,011,138 incorporated by reference in its entirety herein. The subunit structures and three dimensional configurations of different classes of immunoglobulins are well known. The light chains of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains.

A monoclonal antibody is an antibody obtained from a population of substantially homogeneous antibodies, *i.e.,* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which include different antibodies directed against different epitopes, each monoclonal antibody is directed against a single epitope on the antigen. Monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature*, 256:495 (1975), or may be made by recombinant DNA methods (*see e.g.,* U.S. Patent No. 4,816,567). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques

described in Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.,* 222:581-597 (1991), for example.

Antibodies useful in the disclosed methods include chimeric antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chains is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. (USA),* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include PRIMATIZED® antibodies comprising variable domain antigen-binding sequences derived from a non-human primate *(e.g.,* monkey, ape, *etc.*) and human constant region sequences, for example human constant regions derived from IgG1, IgG2, IgG3, or IgG4 isotypes.

Humanized forms of non-human *(e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies are human immunoglobulins (recipient antibodies) in which residues of the hypervariable region are replaced by residues from a hypervariable region of an antibody from (donor antibody) a non-human species antibody, such as an antibody from a mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are also replaced by corresponding non-human residues. Furthermore, humanized antibodies may include residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will include substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will include at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, *see* Jones *et al., Nature* 32 1:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

Papain digestion of antibodies produces two identical antigen-binding fragments, called Fab fragments, each with a single antigen-binding site, and a residual Fc fragment, whose name

reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen. The Fab fragment also contains the constant domain of the light chain and the first constant domain (CHI) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CHI domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. Other chemical couplings of antibody fragments are also known.

Fv is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

Single-chain Fv or scFv antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. For example, the Fv 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 scFv, *see* Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

Diabodies are small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, European Patent No. 404,097; PCT International Publication No. WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. (USA)*, 90:6444-6448 (1993).

An immunoregulatory antibody is an antibody that elicits an effect on the immune system by a mechanism different from depletion of activated B cells. Examples thereof include antibodies that inhibit T cell immunity, B cell immunity, *e.g.,* by inducing tolerance (anti-CD40L, anti-CD40) or other immunosuppressant antibodies (anti-CD80 or B7.1, anti-CD86 or B7.2, or anti-CD4). In some instances, the immunoregulatory antibody of immune cells may also possess the ability to induce or potentiate apoptosis.

Antibodies which specifically bind B7 antigen, such as the B7.1 (CD80), B7.2 (CD86) and B7.3 antigens, are known in the art. Representative B7 antibodies include human and humanized anti-CD80 antibodies, as well as PRIMATIZED® antibodies disclosed by Anderson *et al.* in U.S. Patent No. 6,113,198, as IDEC-114 or 16C10 (also called galiximab), which is a PRIMATIZED® anti-CD80 IgG_1 lambda monoclonal antibody (mAb) containing human constant regions and primate (cynomolgus macaque) variable regions. This antibody binds specifically to human CD80 (B7.1), which is a membrane-associated 60 KDa glycoprotein expressed an activated B cells, activated antigen presenting cells, and activated T cells. (Freeman *et al.*, *J. Immunol.* 1043:2714-22 (1989); Razi-Wolf *et al.*, *Proc. Natl. Acad. Sci. (USA)* 89:4210-4 (1992); and Azuma *et al.*, *J. Exp. Med.* 177:845-50 (1993)). The nucleotide and amino acid sequences of the variable heavy chain and light chain variable regions of IDEC-114 are disclosed in U.S. Patent No. 6,113,898, incorporated herein by reference in its entirety, which are identified therein as the 16C10 and heavy chain and light chain variable regions sequences. The hybridoma which produces the 16C10 antibody was deposited on May 29, 1996, with the American Type Culture Collection (ATCC), currently located at 10801 University Boulevard, Manassas, VA 20110-2209, under the provision of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure ("Budapest Treaty"). The ATCC has assigned hybridoma 16C10 the ATCC Accession No. HB-12119. Additional useful variants of the 16C10 antibody that may be used in the disclosed combination therapies include antibodies that compete with 16C10 for binding to human CD80 antigen, antibodies that bind to the same epitope on human CD80 antigen as 16C10, antibodies having the six complementarity determining regions (CDRs) of 16C10, and antibodies that have the same antigen binding domain as 16C10 (*i.e.*, those residues that are responsible for binding to and/or directly contact CD80 antigen upon binding).

A B cell depleting antibody is an antibody or fragment that binds to a B cell marker and upon administration results in demonstrable B cell depletion. Typically within about several days or less, a B cell depleting antibody will result in a depletion of B cell number by about 50% or more. Depletion may be achieved via various mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC), inhibition of B cell proliferation, and/or induction of B cell death (*e.g.,* via apoptosis).

A B cell surface marker is an antigen expressed on the surface of a B cell which can be targeted with a specific binding partner which binds thereto. Representative B cell surface markers include the CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85 and CD86 (B7.2) leukocyte surface markers. A B cell surface marker is differentially expressed on B cells compared to other non-B cell tissues of a mammal and may be expressed on both precursor B cells and mature B cells. For example, CD20 and CD19 are B cell markers expressed on B cells throughout differentiation of the lineage from the stem cell stage up to a point just prior to terminal differentiation into plasma cells.

The CD20 antigen is a 35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. Other names for CD20 known in the art include B-lymphocyte-restricted antigen and Bp35. The CD20 antigen is described in Clark *et al. Proc. Nat. Acad. Sci. (USA)* 82:1766(1985).

Representative anti-CD20 B cell depleting antibodies include RITUXAN® (a chimeric anti-CD20 antibody) and anti-CD20 antibodies having substantially the same or greater cell depleting activity. This antibody has been demonstrated to provide about 90% of B cell depletion within 24 hours of administration in an effective amount. The nucleotide and amino acid sequence for RITUXAN®, also called rituximab, are disclosed in U.S. Patent No. 5,736,137, the contents of which are incorporated herein by reference in their entirety. Additionally, a CHO cell transfectoma TCAE8 that expresses rituximab has been deposited with the American Type Culture Collection (ATCC) currently located at 10801 University Boulevard, Manassas, VA 20110-2209, under the provision of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure ("Budapest Treaty") and

accorded ATCC deposit number 69119. Rituximab is approved for treatment of relapsed or refractory, low-grade or follicular Lymphoma. Additional useful variants of rituximab that may be used in the disclosed combination therapies include antibodies that compete with rituximab for binding to human CD20 antigen, antibodies that bind to the same epitope on human CD20 antigen as rituximab, antibodies having the six complementarity determining regions (CDRs) of rituximab, and antibodies that have the same antigen binding domain as rituximab (*i.e.*, those residues that are responsible for binding to and/or directly contact CD20 antigen upon binding).

Additional representative anti-CD20 antibodies useful in the disclosed methods include, yttrium-[90]-labeled 2B8 murine antibody Y2B8 (U.S. Patent No. 5,736,137, expressly incorporated herein by reference); murine IgG2a B1, optionally labeled with ^{131}I (BEXXAR™) (U.S. Patent No. 5,595,721, expressly incorporated herein by reference); murine monoclonal antibody 1F5 (Press *et al. Blood* 69(2):584-591 (1987); and chimeric 2H7 antibody (U.S. Patent No. 5,677,180, expressly incorporated herein by reference).

The CD19 antigen is a 90 kDa antigen specifically bound by, for example, by the HD237-CD19 or B4 antibody (Kiesel *et al. Leuk. Res. II,* 12: 1119 (1987)). Like CD20, CD19 is found on cells throughout differentiation of the lineage from the stem cell stage up to a point just prior to terminal differentiation into plasma cells. Binding of a CD19 binding partner may cause internalization of the CD19 antigen. Representative anti-CD199 antibodies have measurable B cell depleting activity, such as at least about 10% the B cell depleting activity of RITUXAN®, or greater B cell depleting activity when administered in the same amount and conditions as RITUXAN®.

The CD22 antigen (also known as BL-CAM and LybB) is expressed on B cells, and is involved in B cell signaling and adhesion. (*See* Nitschke *et al., Curr. Biol.* 7:133 (1997); Stamenkovic *et al., Nature* 345:74 (1990)). This antigen is a membrane immunoglobulin-associated antigen that is tyrosine phosphorylated when membrane Ig is ligated. (Engel *et al., J. Etyp. Med.* 181(4):1521, 1586 (1995)). Representative anti-CD22 antibodies have measurable B cell depleting activity, such as at least about 10% the B cell depleting activity of RITUXAN®, or greater B cell depleting activity when administered in the same amount and conditions as RITUXAN®. Specific examples of antibodies which bind CD22 include LYMPHOCIDE™.

CD23 is the low affinity receptor for IgE expressed by B cells and other cells. CD23 antibodies are known in the art, including, for example PRIMATIZED® 5E8 (also called IDEC-152 or lumiliximab) antibodies specific to human CD23 as described in U.S. Patent No. 6,011,138, issued on July 4, 1999. Additional useful variants of lumiliximab that may be used in the disclosed combination therapies include antibodies that compete with lumiliximab for binding to human CD23 antigen, antibodies that bind to the same epitope on human CD23 antigen as lumiliximab, antibodies having the six complementarity determining regions (CDRs) of lumiliximab, and antibodies that have the same antigen binding domain as lumiliximab (*i.e.*, those residues that are responsible for binding to and/or directly contact CD23 antigen upon binding). Other anti-CD23 antibodies and antibody fragments include those reported by Bonnefoy *et al.,* No. 96 12741; Rector *et al. J. Immunol.* 55:481-488 (1985); Flores-Rumeo *et al. Science* 241:1038-1046 (1993); Sherr *et al. J. Immunol.,* 142:481-489 (1989); and Pene *et al., Proc. Natl. Acad. Sci. (USA)* 85:6820-6824 (1988).

IV.B. Enhancement Of a Therapeutic Effect Of A Therapeutic Antibody Using A HeteroCyclic CD80 Antagonist

As described herein, a heterocyclic CD80 antagonist may be used in combination with a therapeutic antibody to thereby elicit a synergistic therapeutic and/or anti-cancer effect. The enhancement of a therapeutic effect may be assessed using any one or more of the clinical outcomes or tumor microenvironment changes described above with respect to assessing a therapeutic or anti-cancer effect of a heterocyclic compound as a single agent. In addition, a therapeutic or anti-cancer effect of a combination therapy may be demonstrated as enhancement of a particular effect of a therapeutic antibody, for example, induction of antibody-dependent cell-mediated cytotoxicity, complement dependent cytotoxicity, or apoptosis.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a cell mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.,* peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils;) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. NK cells express FcγRIII only, and monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). To assess ADCC activity of a heterocyclic CD80

antagonist, or other anti-cancer agents described herein, an *in vitro* ADCC assay may be preformed, such as that described in U.S. Patent No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. ADCC activity may also be assessed *in vivo, e.g.,* in a animal model such as that disclosed in Clynes *et al. Proc. Natl. Acad. Sci. (USA)* 95:652-656 (1998). *See also* Example 1.

Complement dependent cytotoxicity (CDC) is the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (Clq) to a molecule (*e.g.,* compound of formula (I) or an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.,* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed. *See also* Example 2.

IV.C. Production of Antibodies

A description follows as to representative techniques for the production of the antibodies that may be used in combination with heterocyclic compounds of formula (I) for treating CD80-positive malignancies. Antibodies may be prepared having the immunoregulatory B cell depleting and/or B cell targeting functions as described herein.

(i) Polyclonal antibodies

Polyclonal antibodies are raised for example, in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.,* keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOC l_2, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.,* 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites.

Seven to fourteen days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. For example, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.,* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier monoclonal indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as herein above described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro.* Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk

Institute Cell Distribution Center of San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia, USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:300 1 (1984); Brodeur *et al.,* Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. For example, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the 30 Scatchard analysis of Munson *et al., Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPML-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.,* by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as one source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et*

al., Curr. Opinion in Immunol., 5:256-262 (1993) and Pluckthun, *Immunol. Revs.,* 130:151-188 (1992).

Antibodies or antibody fragments can also be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature,* 348:552-554(1990). Clackson *et al., Nature,* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.,* 21:2265-2266 (1993)).

The DNA encoding antibodies useful in the invention also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. Sci. (USA),* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-binding site having specificity for an antigen and another antigen-binding site having specificity for a different antigen.

(iii) Humanized antibodies

Humanized antibodies may be prepared using any one of a variety of methods including veneering, grafting of complementarity determining regions (CDRs), grafting of abbreviated CDRs, grafting of specificity determining regions (SDRs), and Frankenstein assembly, as described below.

Veneering is based on the concept of reducing potentially immunogenic amino acid sequences in a rodent or other non-human antibody by resurfacing the solvent accessible exterior of the antibody with human amino acid sequences. Thus, veneered antibodies appear less foreign to human cells. *See* Padlan, *Mol. Immunol.* 28:489-98, (1991). A non-human antibody is veneered by (1) identifying exposed exterior framework region residues in the non-human antibody, which are different from those at the same positions in framework regions of a human

antibody, and (2) replacing the identified residues with amino acids that typically occupy these same positions in human antibodies.

Grafting of CDRs is performed by replacing one or more CDRs of an acceptor antibody (*e.g.*, a human antibody) with CDRs of a donor antibody (*e.g.*, a non-human antibody). Acceptor antibodies may be selected based on similarity of framework residues between a candidate acceptor antibody and a donor antibody and may be further modified to introduce similar residues. For example, a human acceptor framework may comprise a heavy chain variable region of a human sub-group I consensus sequence, optionally with non-human donor residues at one or more positions. As another example, a human acceptor framework may comprise a light chain variable region of a human sub-group I consensus sequence, optionally with non-human donor residues at one or more positions. Following CDR grafting, additional changes may be made in the donor and/or acceptor sequences to optimize antibody binding and functionality. *See e.g.*, PCT International Publication No. WO 91/09967.

Grafting of abbreviated CDRs is a related approach. Abbreviated CDRs include the specificity-determining residues and adjacent amino acids, including those at positions 27d-34, 50-55 and 89-96 in the light chain, and at positions 31-35b, 50-58, and 95-101 in the heavy chain (numbering convention of (Kabat *et al.*, (1987)). *See* Padlan *et al.*, *FASEB J.* 9: 133-9 (1995). Grafting of specificity-determining residues (SDRs) is premised on the understanding that the binding specificity and affinity of an antibody combining site is determined by the most highly variable residues within each of the complementarity determining regions (CDRs). Analysis of the three-dimensional structures of antibody-antigen complexes, combined with analysis of the available amino acid sequence data was used to model sequence variability based on structural dissimilarity of amino acid residues that occur at each position within the CDR. *See* Padlan *et al.*, *FASEB J.* 9: 133-139 (1995). Minimally immunogenic polypeptide sequences consisting of contact residues, which are referred to as specificity-determining residues (SDRs), are identified and grafted onto human framework regions.

According to the Frankenstein approach, human framework regions are identified as having substantial sequence homology to each framework region of the relevant non-human antibody, and CDRs of the non-human antibody are grafted onto the composite of the different human framework regions. A related method also useful for preparation of antibodies of the invention is described in U.S. Patent Application Publication No. 2003/0040606.

(iv) Human antibodies

Human antibodies can be generated in transgenic animals *(e.g.,* mice) capable of producing human antibodies in the absence of endogenous immunoglobulin production. For example, homozygous deletion of the antibody heavy-chain joining region PH gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ line mutant mice will result in the production of human antibodies upon antigen challenge. *See, e.g.,* Jakobovits *et al., Proc. Natl. Acad. Sci. (USA),* 90:255 1 (1993); Jakobovits *et al., Nature,* 362:255-258 (1993); Bruggermann *et al., Year in Immunol.,* 7:33 (1993); and U.S. Patent Nos. 5,591,669; 5,589,369; and 5,545,807.

Alternatively, phage display technology (McCafferty *et al., Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro,* from immunoglobulin variable (V) domain gene repertoires from non-immunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review *see, e.g.,* Johnson *et al., Cur. Op. Stru. Biol.* 3:564-57 1 (1993). Several sources of V gene segments can be used for phage display. Clackson *et al., Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self antigens) can be isolated essentially as described by Marks *et al., J. Mol. Biol,* 222:581-597 (1991), or Griffith *et al., EMBO J.* 12:725-734 (1993). *See also,* U.S. Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by *in vitro* activated B cells (*see* U.S. Patent Nos. 5,567,610 and 5,229,275).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. These fragments may be derived via proteolytic digestion of intact antibodies or by recombinant production *(see, e.g.,* Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science,* 229:81 (1985)). For example, the antibody fragments can be isolated from antibody phage libraries, as described above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al., Bio/Technology* 10: 163-167 (1992)).

According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments are known in the art, for example, techniques for production of a single chain Fv fragment (ScFv), as described in PCT International Publication No. WO 93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. The antibody fragment may also be a linear antibody, *e.g.,* as described in U.S. Patent No. 5,641,870.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Representative bispecific antibodies may bind to two different epitopes of the B cell surface marker. Other such bispecific antibodies may bind a first B cell marker and further bind a second B cell surface marker. Other bispecific antibodies may bind to a B cell marker and to a leukocyte marker such as a T cell receptor marker (*e.g.,* CD2 or CD3), or Fc receptors for IgG (FcR), such as FcRI (CD64), FcRII (CD32) and FcRIII (CD16). Bispecific antibodies may also be used to localize cytotoxic agents to the B cell. These antibodies bind to a B cell marker and to a cytotoxic agent (*e.g.,* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.,* $F(ab)_2$ bispecific antibodies).

Techniques for making bispecific antibodies are known in the art. Full length bispecific antibodies may be produced by coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of

which only one has the correct bispecific structure. *See e.g.*, PCT International Publication No. WO 93/08829, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

In one example, bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity, and a hybrid immunoglobulin heavy chain light chain pair with a second binding specificity. This asymmetric structure mass facilitate the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as described in PCT International Publication No. WO 94/04690.

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The interface includes at least a part of the CH3 domain of an antibody constant domain and one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.,* tyrosine or tryptophan). Compensatory cavities of identical or similar size to the large side chains are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.,* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or heteroconjugate antibodies. For example, one of the antibodies in a heteroconjugate can be coupled to avidin, the other to biotin. *See e.g.,* U.S. Patent No. 4,676,980, PCT International Publication Nos. WO 91/00360 and WO 92/200373, and European Patent No. 03089. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are representative agents disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Bispecific antibodies may also be prepared using chemical linkage. Brennan *et al., Science,* 229:81(1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody.

Fab'-SH fragments may also be directly recovered from *E. coli*, and then chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.,* 175:2 17-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The resultant bispecific antibody was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. Biospecific antibody fragments may also be prepared using the diabody technology described by Hollinger *et al., Proc. Natl. Acad. Sci. (USA)*, 90:6444-6448 (1993). The fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. *See* Gruber *et al., J. Immunol.*, 152:5368 (1994). For further details of generating bispecific antibodies *see*, for example, Suresh *et al., Methods. in Enz.,* 121:210 (1986).

Antibodies with more than two valencies are also useful for combination therapies comprising administration of a heterocyclic CD80 antagonist and at least one other anti-cancer agent. For example, trispecific antibodies are described by Tutt *et al. J. Immunol.* 147: 60(1991).

V. Pharmaceutical Formulation & Dose

Heterocyclic compounds of formula (I) and other therapeutic agents of the invention are readily prepared and formulated for safe and efficacious clinical use. Suitable formulations for administration to a subject include aqueous and non-aqueous sterile injection solutions which

may contain anti-oxidants, buffers, bacteriostats, antibacterial and antifungal agents (*e.g.*, parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal), solutes that render the formulation isotonic with the bodily fluids of the intended recipient (*e.g.*, sugars, salts, and polyalcohols), suspending agents and thickening agents. Suitable solvents include water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), and mixtures thereof.

A compound of formula (I), optionally in combination with other anti-cancer agents, may be formulated in a pharmaceutically acceptable carrier, for example, large slowly metabolized macromolecules such as proteins, polypeptides, liposomes, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles. Pharmaceutically acceptable salts may also be used, for example, mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulfates, or salts of organic acids, such as acetates, propionates, malonates and benzoates. Formulations may additionally contain liquids such as water, saline, glycerol, and ethanol, and/or auxiliary substances, such as wetting or emulsifying agents or pH buffering substances, may be present in such compositions. Such carriers enable the compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

The CD80 antagonists disclosed herein may also be formulated as liposomes. Liposomes containing the antagonist are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang *et al., Proc. Natl. Acad. Sci. (USA),* 77:4030 (1980); U.S. Patent. No's. 4,485,045 and 4,544,545; and PCT International Publication No. WO 97/38731. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

A liposome is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the antagonists disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of an antibody of the present

invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. *See* Gabizon *et al. J. National Cancer Inst.* 81(19)1484 (1989).

The active ingredients may also be entrapped in microcapsules prepared, for example, by 30 coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly- (methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A., ed. (1980).

Sustained-release preparations may also be prepared. Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, *e.g.,* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl- methacrylate), or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, noir degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use for administration to a subject or for subsequent radiolabeling with an isotope appropriate for the intended application.

A stable lyophilized formulation of a heterocyclic compound of formula (I) or a heterocyclic compound of formula (I) in combination with another anti-cancer agent, may be prepared by (a) dissolving an antibody/drug conjugate to a final concentration of 0.5 to 2 mg/ml in a solution comprising a cryoprotectant at a concentration of 1.5%-5% by weight, a polymeric bulking agent at a concentration of 0.5-1.5% by weight, electrolytes at a concentration 0.01 M to 0.1 M, a solubility facilitating agent at a concentration of 0.005% to 0.05% by weight, buffering agent at a concentration of 5-50 mM such that the final pH of the solution is 7.8-8.2, and water;

(b) dispensing the above solution into vials at a temperature of +5°C to +10°C; (c) freezing the solution at a freezing temperature of -35°C to -50°C; (d) subjecting the frozen solution to an initial freeze drying step at a primary drying pressure of 20 to 80 microns at a shelf temperature at -10°C to -40°C for 24 to 78 hours; and (e) subjecting the freeze-dried product of step (d) to a secondary drying step at a drying pressure of 20 to 80 microns at a shelf temperature of +10°C to + 35°C for 15 to 30 hours.

Representative cryoprotectants useful for lyophilization of the cryoprotectant include alditol, mannitol, sorbitol, inositol, polyethylene glycol, aldonic acid, uronic acid, aldaric acid, aldoses, ketoses, amino sugars, alditols, inositols, glyceraldehydes, arabinose, lyxose, pentose, ribose, xylose, galactose, glucose, hexose, idose, mannose, talose, heptose, glucose, fructose, gluconic acid, sorbitol, lactose, mannitol, methyl α-glucopyranoside, maltose, isoascorbic acid, ascorbic acid, lactone, sorbose, glucaric acid, erythrose, threose, arabinose, allose, altrose, gulose, idose, talose, erythrulose, ribulose, xylulose, psicose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine, sucrose, trehalose, neuraminic acid, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans, levan, fucoidan, carrageenan, galactocarolose, pectins, pectic acids, amylose, pullulan, glycogen, amylopectin, cellulose, dextran, pustulan, chitin, agarose, keratin, chondroitin, dermatan, hyaluronic acid, alginic acid, xanthan gum, starch, sucrose, glucose, lactose, trehalose, ethylene glycol, polyethylene glycol, polypropylene glycol, glycerol and pentaerythritol.

For example, the cryoprotectant sucrose may be used at a concentration of 1.5% by weight, the polymeric bulking agent Dextran 40 or hydroxyethyl starch 40 may be used at a concentration of 0.9% by weight, the electrolyte used in the lyophilization solution is sodium chloride, which is present at a concentration of 0.05 M, and the buffering agent tromethamine may be used at a concentration of 0.02 M. A solubility facilitating agent (*e.g.*, a surfactant such as Polysorbate 80) may also be used during the lyophilization process. Usually this solubility facilitating agent is a surfactant. Representative steps for preparation of a lyophilized formulation include freezing the vials at a temperature of -45 °C; the frozen solution is subjected to an initial freeze drying step at a primary drying pressure of 60 microns and at a shelf temperature of -30°C for 60 hours; and subjecting the freeze-dried product to a secondary drying step at a drying pressure of 60 microns at a shelf temperature of +25°C for 24 hours.

When used for combination therapies, the heterocyclic compounds of formula (I) and one or more additional therapeutic agent (*e.g*, immunoregulatory antibody, B cell depleting antibodies, other anti-cancer agents, *etc.*) may be in the same formulation or in different formulations. Administration can be concurrent or sequential, and is effected in either order. Such administration may be effected by repeated administration of both compound of formula (I) and the antibodies, for a prolonged period of time.

The compound of formula (I) and other therapeutic agents may be administered parenterally, for example, via intravascular, subcutaneous, intraperitoneal, or intramuscular administration. For delivery of compositions to pulmonary pathways, compositions may be administered as an aerosol or coarse spray, *i.e.* transnasal administration. Intrathecal or intramedullary administration may be used for treatment of central nervous system (CNS) and CNS-related cancers. The compound of formula (I) and other therapeutic agents of the invention may also be administered transdermally, transcutaneously, topically, enterally, intravaginally, sublingually or rectally. A delivery method is selected based on considerations such as the condition and site to be treated, the type of antibody formulation, and the therapeutic efficacy of the composition. Intravenous administration may be routinely used in the clinic.

The present invention provides that an effective amount of a compound of formula (I) either alone or in combination with other therapeutic antibodies/agents is administered to a subject. The term subject for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* An effective amount is an amount sufficient to elicit a desired biological response. For example, when administered to a cancer-bearing subject, an effective amount is an amount sufficient to elicit an anti-cancer activity, including cancer cell cytolysis, inhibition of cancer cell proliferation, induction of cancer cell apoptosis, reduction of cancer cell antigens, delayed tumor growth, and inhibition of metastasis. Tumor shrinkage is well accepted as a clinical surrogate marker for efficacy. Another well accepted marker for efficacy is progression-free survival.

As a general proposition, the administration of compound of formula (I) is carried out by administering an effective amount to a patient in need of such treatment or prophylaxis. Actual dosage levels of active ingredients in a composition of the invention may be varied so as to administer an amount of the composition that is effective to achieve the desired diagnostic or

therapeutic outcome. Administration regimens may also be varied. A single injection or multiple injections may be used. The combined dosage level and regimen will depend upon a variety of factors including the activity and stability (*i.e.*, half life) of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, the disease or disorder to be detected and/or treated, and the physical condition and prior medical history of the subject being treated.

Further, as discussed above, the formulation may contain additional therapeutic agents other than a heterocyclic compound of formula (I). The therapeutically effective amount of a therapeutic antibody administered parenterally per dose will typically be in the range of about 0.1 to 500 mg/kg of patient body weight per day, with the typical initial range of antagonist used being in the range of about 2 to 100 mg/kg. For example, an anti-CD80 antibody such as IDEC-114 may be administered at a dosage in the range from about 0.5 mg/kg body weight to 10 mg/kg body weight, or a dosage in the range from about 0.05 mg/kg body weight to 100 mg/kg body weight. A B cell depleting antibody such as RITUXAN® may be administered at a dosage of from about 20mg/m^2 to about 1000mg/m^2. For example, one may administer to the patient one or more doses of substantially less than 375mg/m^2 of the antibody, *e.g.,* where the dose is in the range from about 20mg/m^2 to about 250mg/m^2, for example from about 50mg/m^2 to about 200mg/m^2.

For a radiolabeled conjugate, an effective dose is typically in the range from about 1 mCi to about 300 mCi, normally about 5 mCi to 100 mCi, depending on the radioisotope and the binding affinity of the antibody. For scintigraphic imaging using radioisotopes, typical doses of a radioisotope may include an activity of about 10 μCi to 50 mCi, or about 100 μCi to 25 mCi, or about 500 μCi to 20 mCi, or about 1 mCi to 10 mCi, or about 10 mCi.

The therapeutically effective dose of the present invention may be estimated initially either in cell culture assays or in animal models, usually in rodents, rabbits, dogs, pigs, and/or or primates. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information may then be used to determine useful doses and routes for administration in humans. Typically a minimal dose is administered, and the dose is escalated in the absence of dose-limiting cytotoxicity. Determination and adjustment of an effective amount or dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art of medicine.

For additional guidance regarding formulation, dose, administration regimen, and measurable therapeutic outcomes, *see* Berkow et al. (2000) The Merck Manual of Medical Information, Merck & Co., Inc., Whitehouse Station, New Jersey; Ebadi (1998) CRC Desk Reference of Clinical Pharmacology, CRC Press, Boca Raton, Florida; Gennaro (2000) Remington: The Science and Practice of Pharmacy, Lippincott, Williams & Wilkins, Philadelphia, Pennsylvania; Katzung (2001) Basic & Clinical Pharmacology, Lange Medical Books / McGraw-Hill Medical Pub. Div., New York; Hardman et al. (2001) Goodman & Gilman's the Pharmacological Basis of Therapeutics, The McGraw-Hill Companies, Columbus, Ohio; Speight & Holford (1997) Avery's Drug Treatment: A Guide to the Properties, Choices, Therapeutic Use and Economic Value of Drugs in Disease Management, Lippincott, Williams, & Wilkins, Philadelphia, Pennsylvania.

EXAMPLES

The following examples have been included to illustrate modes of invention. Certain aspects of the following examples are described in terms of techniques and procedures found or contemplated by the inventor to work well in the practice of the invention. These examples illustrate standard laboratory practices of the inventor. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following examples are intended to be exemplary only and that numerous changes, modifications, and alterations may be employed without departing from the scope of the invention.

EXAMPLE 1

Enhancing Antibody-Dependent Cellular Cytotoxicity (ADCC) in CD80-Positive Malignant Cells Using Heterocyclic Compounds In Combination With Therapeutic Antibodies

Heterocyclic compounds of formula (I) are prepared essentially as described in U.S. Patent No. 7,081,456, the contents of which are incorporated by reference in its entirety herein. Additional representative techniques are described by Erbe *et al.*, *J. Biol. Chem.* 277(9): 7363-68 (2002), also incorporated herein by reference in its entirety.

The effect of combining a compound of formula (I) with an anti-CD80 antibody or anti-CD20 antibody to increase host effector mediated killing of tumor cells is determined. In such experiments, a fixed concentration of compound of formula (I) is combined with varying concentrations of anti-CD80 antibody or anti-CD20 to determine effective tumor killing.

To determine the ADCC activity, CD20-expressing and CD80-expressing B cell lymphoma cell lines (SKW, SB, and Daudi cells) are cultured in complete medium. Complete medium is RPMI 1640 medium (Irvine Scientific of Santa Ana, California) supplemented with 10% heat inactivated FBS (Hyclone), 2 mM l-glutamine, 100 units/ml of penicillin, and 100 ug/ml of streptomycin. The SKW cell line is Epstein-Barr virus (EBV) positive and can be induced to secrete IgM (SKW 6.4 available from ATCC). The SB cell line originated from a patient with acute lymphoblastic leukemia and is positive for EBV (CCL-120 available from ATCC). The Daudi cell line is isolated from a patient with Burkitt's lymphoma (CCL-213 available from ATCC). CD80-expressing Chinese hamster ovary cells (CHO) are generated by recombination expression.

In the ADCC assay, SKW or SB cells and activated human peripheral monocytes (PBMC) are used as targets and effector cells, respectively. PBMC are isolated from whole blood of healthy donors using Histopaque (Sigma-Aldrich Corporation of St. Louis, Missouri). The PBMC are cultured at a concentration of 5 x 10^6 cells/ml in complete medium with 20 U/ml recombinant human IL-2 (Invitrogen of Carlsbad, California) in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2. After overnight culture, 1 x 10^6 SKW or SB target cells are labeled with 150 µCi of ^{51}Cr (Amersham Pharmacia Biotech of Piscataway, New Jersey) for 1 hour at 37°C and 5% CO_2. The cells are washed four times and resuspended in 5 ml of complete medium; 50 µl of cell suspension is dispensed into each well containing equal volume of test or control compound and antibodies.

To determine the ADCC activity, an anti-cancer agent, such as Rituximab or IDEC-114, is used alone or in simultaneous or sequential combination with a heterocyclic compound of formula (I). The following controls are also included in triplicate: target cells incubated with 100 µl complete medium to determine spontaneous release and target cells incubated with 100 µl 0.5% TRITON X®-100 (Sigma-Aldrich Corporation) to determine maximum release. The culture is incubated for 4 hours at 37°C and 5% CO_2 and the ^{51}Cr released in the culture

supernatant due to cell lysis is determined by a gamma counter. The cytotoxicity is expressed as the percentage of specific lysis and calculated as follows:

$$\frac{^{51}\text{Cr release of test samples - spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release - spontaneous } ^{51}\text{Cr release}} \quad (\text{X } 100)$$

EXAMPLE 2

Enhancing Complement Dependent Cytotoxicity (CDC)

in CD80-Positive Malignant Cells Using A Heterocyclic CD80 Antagonist

In Combination With Therapeutic Antibodies

The ability of a compound of formula (I) to enhance CDC of CD80 positive malignancies is determined. To determine the CDC activity, an anti-cancer agent, such as Rituximab or IDEC-114, is used alone or in simultaneous or sequential combination with a heterocyclic compound of formula (I). In such experiments, a fixed concentration of a compound of formula (I) is combined with varying concentrations of anti-CD80 antibody or anti-CD20 to determine effective tumor killing.

CDC activity is determined using B cell Lymphoma cell lines, as described in Example 1. Dilutions of antibodies are made at 4x concentration and 50 µl is dispensed into each well of 96 well plates. The SKW or Daudi cells are labeled with ^{51}Cr (150 µCi/10^6 cells) for 1 hour at 37°C and 5% CO_2. The cells are washed four times and resuspended in complete medium, and 1 x 10^4 cells in 50 µl is dispensed into each well. One hundred (100) µl of normal human serum complement (Quidel of San Diego, California) diluted 1:4 or 1:8 in complete medium is added. Controls for determining spontaneous and maximum release and set up are described in Example 1. The cultures are incubated 4 hours at 37°C and 5% CO_2. The radioactivity released into the culture supernatant is determined by a gamma counter. The cytotoxicity is expressed as the percentage of specific lysis and is calculated as described above.

EXAMPLE 3

In Vivo Therapeutic Effect Of A Heterocyclic CD80 Antagonist Alone Or In Combination With Other Anti-Cancer Agents

A human lymphoma tumor model in severe immunodeficiency (SCID) mice is developed. Human SKW lymphoma cells are inoculated intravenously (IV) into BALB/c SCID mice. After inoculation, SKW cells disseminate throughout the mouse and grow primarily in the lungs and liver. Briefly, 3 x 10^6 to 4 x 10^6 SKW cells are intravenously injected into 6- to 8-week old SCID mice and their survival is monitored for 45 to 60 days.

Treatment with a compound of formula (I) is initiated 1 day after tumor inoculation and repeated as needed to administer an effective dose. A subset of mice additionally receive an effective dose of a second anti-cancer agent (*e.g.*, C2B8/rituximab or IDEC-114/galiximab). Control animals receive vehicle only and/or isotype matched non-therapeutic antibody Anti-cancer activity and toxicity are monitored daily or on an ongoing basis.

EXAMPLE 4

Inhibition Of CD80-CD28 Binding But Not CD80-CTLA4 Binding By A Heterocyclic CD80 Antagonist

The ability of a compound of formula (I) to inhibit the interaction of CD80 with CD28, without inhibiting the interaction of CD80 with CTLA4 is determined essentially as described by Greene *et al.*, *J. Biol. Chem.*, 271(43): 26762-26771 (1996). Recombinant forms of the extracellular domains of CD28 and CTLA-4Ig fusion proteins are prepared that contain a synthetic thrombin cleavage site between the extracellular domains and the Fc regions. Polymerase chain reaction products encoding the extracellular domains of CD28 and CTLA4Ig are digested with *Hin*dIII and *Bcl*I restriction enzymes and ligated into a *Hin*dIII/*Bam*HI-digested Ig expression vector encoding a synthetic thrombin cleavage site 5' to the hinge region of a human IgG1 Fc domain. The resulting thrombin cleavage site-containing fusion constructs are designated CTLA4tIg and CD28tIg, respectively. Ig fusion proteins are produced by transiently transfected COS cells or stably transfected Chinese hamster ovary cells and purified by affinity

chromatography on immobilized protein A-Sepharose (Repligen of Cambridge, Massachusetts, USA).

The fusion proteins are digested with purified bovine thrombin (Armour Pharmaceutical Corporation of Kankakee, Illinois, USA) at a final concentration of 5 units/mg of protein at 37 °C for 40-60 min. The cleaved extracellular domains are purified from other digestion products and are designated CTLA-4tp, and CD28tp, respectively. The predicted amino acid sequence of CTLA-4tp contains residues 1-127 (numbered as in Peach, R. J., *et al.*, *J. Exp. Med.* 180: 2049-2058 (1994)) of CTLA-4 fused to a thrombin-cleaved linker peptide (PDSDpgggggrlv, where lower-case letters denote linker sequences). CD28tp contains residues 1-134 of CD28 fused to a thrombin-cleaved linker (-PSKPdpgggggrlv-).

CD28tp or CTLA-4tp is immobilized in 96 well plates by coating the wells with a solution containing either CD28tp or CTLA-4tp for 16-24 hours. Following this coating, the wells are washed with a blocking solution, preferably containing at least 0.1% BSA. CD20-expressing and/or CD80-expressing B cell lymphoma cell lines (SKW, SB, and Daudi cells, labeled with 150 μCi of ^{51}Cr, as described in Example 1) are added to the 96 well plates and incubated for 4 hours at 37°C and 5% CO_2. Negative control wells are not coated with either CD28tp or CTLA-4tp; positive control wells are coated with either CD28tp or CTLA-4tp, with no addition of a compound of formula (I); test wells are coated with either CD28tp or CTLA-4tp, with the addition of various concentrations of a compound of formula(I). Following incubation, the wells are washed 4 times with PBS and incubated with 100 μl 0.5% TRITON X®-100 (Sigma-Aldrich Corporation) to lyse remaining cells. The ^{51}Cr released in the culture supernatant due to cell lysis is determined by a gamma counter. The binding is expressed as a percentage of the positive control wells.

EXAMPLE 5

Induction Of Cytotoxicity Of CD80-Positive Malignant Cells Using A Heterocyclic CD80 Antagonist

Induction of cytotoxicity of CD80-positive malignant cells after 5 days in culture is measured by Alamar Blue, a dye-reduction assay used to identify live cells (*see* Gazzano-Santoro *et al., J. Immunol. Meth.* 202: 163-171 (1997)). Briefly, 1 x 10^5 CD80-positive malignant cells

in growth medium are incubated with varying concentrations of a heterocyclic compound of formula (I) (1×10^{-6} M to 1×10^{-8} M) in cell culture tubes at 37°C. for 4 hours. After incubation, cells are washed, re-suspended in growth medium at 1×10^{5} cells/ml concentration and 200 µl of cell suspension was added to each well of 96-well flat-bottom plate. Plates are incubated at 37°C. and tested for viability at different time points. During the last 18 hours of incubation, 50 µl of redox dye Alamar Blue is added to each well. Following incubation, plates are cooled by incubating at room temperature for 10 minutes on a shaker, and the intracellular reduction of the dye is determined. Fluorescence is read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results are expressed as relative fluorescence units (RFU). The percentage viability is calculated as follows:

[1- (average RFU of test sample ÷ Average RFU of control cells)] x 100%.

Induction of cytotoxicity is observed as reduced viability. Induction of cytotoxicity of CD80-positive cells may also determined in the presence of a compound of formula (I) in combination with an anti-CD80 antibody and/or an anti-CD20 antibody.

EXAMPLE 6

Induction of Apoptosis In CD80-Positive Malignant Cells Using A Heterocyclic CD80 Antagonist

The ability of a compound of formula (I) to induce apoptosis in CD80-positive malignant cells is determined. CD80-positive cells, such as SKW, SB, and Daudi cells are incubated at 0.5×10^{6} cells/ml density in culture media with 10 µg/mL of a compound of formula (I) at 37°C and 5% CO_2. After 1 hour of incubation the excess compound in the media is removed by centrifugation. The cells are resuspended in growth media in appropriate volumes and added into 24 well tissue culture plates (1.5×10^{6} cells/well). Following incubation for 18 hours, cells are harvested and analyzed for apoptosis by flowcytometry. In particular, the cells are washed and fixed at 4°C using CYTOFIX® (CYTOFIX/CYTOPERM® Kit). After 20 minutes of fixation, cells are washed and 15 µl of affinity purified PE-conjugated polyclonal rabbit anti-caspase-3 antibody and 50 µl of CYTOPERM® are added. Cells are incubated on ice in the dark for 30 minutes. After incubation cells are washed once and resuspended in

CYTOPERM®wash. Flow cytometry data is acquired on a flow cytometer and analyzed using standard techniques and software known in the art.

Induction of apoptosis by a compound of formula (I) in the presence of anti-CD80 and/or anti-CD20 antibodies may be similarly determined. CD80-positive cells are incubated as described above with a compound of formula (I) and with an anti-CD80 antibody and/or an anti-CD20 antibody. The excess compound and antibodies are removed by centrifugation. The cells are resuspended in growth media in appropriate volumes and added into 24 well tissue culture plates ($1.5x10^6$ cells/well) with and without the addition of goat anti-human Ig-Fcγ specific secondary antibody (15 μg/ml) as a crosslinker. Following incubation for 18 hours, cells are harvested and analyzed for apoptosis by flow cytometry.

EXAMPLE 7

Treatment Of Relapsed Or Refractory Hodgkin's Lymphoma Patients Using A Heterocyclic CD80 Antagonist

Adult patients (at least 18 years old) with histologically confirmed classical Hodgkin's lymphoma are selected for treatment with the compound of formula (I). Patients have a measurable disease (*e.g.*, at least one lesion $\geq$ 10 mm) and adequate hematologic, renal and hepatic function. Patients are treated with the compound of formula (I) for a period of one month, for example once weekly for 4 weeks. After the administration of the induction therapy as described above, patients receive extended therapy and/or escalating doses until disease regression or toxicity.

Evaluation of disease is performed by comprehensive scans (computed tomography, magnetic resonance imaging, and x-rays) and physical examination at baseline (study entry) using standard outcome measures for clinical trials (complete response, unconfirmed complete response, partial response, stable disease and progressive disease) as defined by the International Workshop Response Criteria for Non-Hodgkin's lymphoma. Relevant end points include overall response rate, complete remission rate, unconfirmed complete remission rate, partial remission rate, duration of response and time to progression. Additional relevant indices of efficacy include changes in the tumor microenvironment that promote tumor immunity, including enhancement of adaptive immune responses, for example, by inhibition of regulatory T cell suppression, a decrease in immunoregulatory cells and/or inflammatory cells in the tumor

microenvironment, down-regulation of cytokines and other factors that support tumor progression, a decrease in tumor cell growth and/or migration, a decrease in tumor vascularization, *etc*. *See e.g.*, Example 8.

EXAMPLE 8

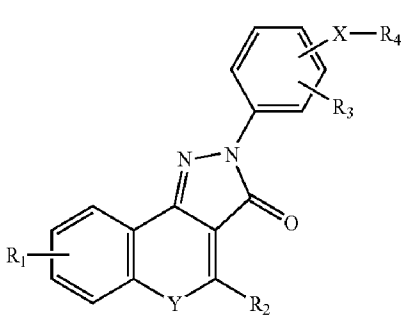
Modulation Of The Tumor Microenvironment Using A Heterocyclic CD80 Antagonist

In patients receiving therapy with a compound of formula (I), serum and/or biopsies are obtained at the following time points: prior to treatment on day 1, in week 4 (completion of induction), in week 8, and every 12 weeks thereafter for the duration that the patient remains on study. Biopsies may be obtained from a tumor mass, tumor nodules, and/or tumor draining lymph nodes. Immune cell functions in the samples are assayed according to methods known in the art. Cytokine levels are determined using antibodies that specifically bind to the cytokine(s) of interest. Regulatory T cells, Th2 cells, and macrophages are quantified using flow cytometric analyses using appropriate molecular markers as known in the art and described herein. For analysis of the tumor microenvironment, malignant cells are first depleted from the sample using an antibody that specifically binds a tumor-associated antigen. One skilled in the art can readily identify an appropriate tumor-associated antigen for the malignancy being treated. Regulatory T cell content is assessed by identifying a population of $CD4^+$, $CD25^{hi}$ cells, optionally in combination with one or more of cytotoxic T lymphocyte-associated antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), CC chemokine receptor 4 (CCR4), forkhead box p3 (FOXP3), CC chemokine receptor 6 (CCR6), CD30, $CD62L^{hi}$, $CD45RB^{lo}$, $CD45RO^{hi}$, and/or $CD45RA^-$. Th2 cells may be identified TCR^+ (T cell receptor) $CD4^+$, $CD25^-$ cells. Macrophages are quantified using biomarkers including TLR5, FCGR1A, SEPT10, LGMN, and/or C3AR1. Macrophages may also be quantified using biomarkers to calculate lymphocyte associated macrophage (LAM) content, for example, as described by Farinha et al., *Blood*, 2005, 106(16):2169-2174. Regulatory T cell proliferation is assayed by culturing of cells in the presence of a vital dye, such as carboxyfluorescein diacetate (CFSE).

CLAIMS

What is claimed is:

1. A method of treating a CD80-positive malignancy in a subject comprising administering to the subject a therapeutically effective amount of a compound of formula (I)



(I)

wherein, R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F; R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl; Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl; X represents a bond or a divalent C_1 C_6 alkylene radical; R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -$(Alk)_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7 cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.

2. The method of claim 1, wherein the CD80-positive malignancy is a B cell malignancy.

3. The method of claim 2, wherein the B cell malignancy is a B cell lymphoma or leukemia.

4. The method of claim 3, wherein the B cell malignancy is a B cell lymphoma.

5. The method of claim 4, wherein the B cell lymphoma is selected from the group consisting of Hodgkin's lymphoma, non-Hodgkin's lymphoma, low grade/follicular non-Hodgkin's lymphoma (NHL), cell lymphoma (FCC), mantle cell lymphoma (MCL), diffuse large cell lymphoma (DLCL), small lymphocyte (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL or Waldenstrom's Macroglobulinemia.

6. The method of claim 3, wherein the leukemia is ALL-L3 (Burkitt's type leukemia), chronic lymphocytic leukemia (CLL) or monocytic cell leukemia.

7. The method of claim 1, wherein R_1 is H, F, Cl, methyl or methoxy.

8. The method of claim 1, wherein R_2 is H, methyl, methoxy, cyclopropyl, phenyl, or fluoro-, chloro, methyl, or methoxy-substituted phenyl.

9. The method of claim 1, wherein R_3 is H, F, Cl, methyl, or methoxy.

10. The method of claim 1, wherein Y is —O—, —S—, or —N(R_5), wherein R_5 represents H or methyl.

11. The method of claim 1, wherein X is a bond, or a —CH_2— or —CH_2CH_2— radical.

12. The method of claim 1, wherein R_4 represents —C(=O)NHR_6, wherein R_6 is a radical of formula -Alk_b-Q, wherein b is 1 and Alk is a — $(CH_2)_n$—, —CH($(CH_2)_mCH_3$)$(CH_2)_n$—, —CH($(CH_2)_mCH_3$) ($(CH_2)_pCH_3$)$(CH_2)_n$—, — $(CH_2)_n$—O— $(CH_2)_m$—, or — $(CH_2)_n$—O— $(CH_2)_n$—O— $(CH_2)_m$—, radical where n is 1, 2, 3 or 4 and m and p are independently 0, 1, 2, 3 or 4, and Q represents H, —OH, —$COOCH_3$ phenyl, cyclopropyl, cyclopentyl, cyclohexyl, pyridyl, furyl, thienyl, or oxazolyl.

13. The method of claim 1, wherein R_1 is H, F, or Cl; R_2 is H; R_3 is H, F, or Cl; Y is —NH—; X is a bond; and R_4 represents —C(=O)NHR_6, wherein: R_6 is a radical of formula -

Alk_b-Q wherein b is 1 and Alk is a —$(CH_2)_n$—, —$CH((CH_2)_mCH_3)(CH_2)_n$—, —$CH((CH_2)_mCH_3)((CH_2)_pCH_3)(CH_2)_n$—, —$(CH_2)_n$—O—$(CH_2)_m$—, or —$(CH_2)_n$—O—$(CH_2)_n$—O—$(CH_2)_m$—, radical where n is 1, 2, 3 or 4 and m and p are independently 0, 1, 2, 3 or 4, and Q represents H, —OH, —$COOCH_3$ phenyl, cyclopropyl, cyclopentyl, cyclohexyl, pyridyl, furyl, thienyl, or oxazolyl.

14. The method of claim 1, wherein R_1 is F, R_2 is H or cyclopropyl, R_3 is H, X is a bond, and R_4 is —$C(=O)NHR_6$.

15. The method of claim 1, wherein the compound of formula (I) induces apoptosis of malignant B cells.

16. The method of claim 1, wherein the compound of formula (I) induces cytotoxicity of malignant B cells.

17. The method of claim 1, wherein the compound of formula (I) is conjugated to a cytotoxin.

18. The method of claim 17, wherein the cytotoxin is a cystotatic agent, an alkylating agent, an anti-metabolite, an anti-proliferative agent, a tubulin binding agent, a hormone, a hormone antagonist, an antibiotic, an enzyme, a radiosensitizing drug, or a cell growth inhibitor.

19. The method of claim 1, wherein the compound of formula (I) is administered parenterally, orally, intraarterially, intramuscularly, rectally, vaginally, or subcutaneously.

20. The method of claim 1, further comprising administering to the subject at least one chemotherapeutic agent, wherein the compound of formula (I) and the at least one chemotherapeutic agent are administered simultaneously or sequentially in either order.

21. The method of claim 20, wherein the at least one chemotherapeutic agent is selected from the group consisting of alkylating agents, vinca alkaloids, vincristine, vinblastine, procarbazine, methotrexate, prednisone, mechlethamine, adriamycin, bleomycin, dacarbazine, chlorambucil, lomustine, doxorubicin, streptozotocin, carmustine, cyclophosphamide, leucovorin, etoposide, cytarabine, cytosine arabinoside, 2-chlorodeoxyadenosine (2-CDA), 2'-deoxycoformycin, fludarabine, cisplatin, ifosfamide, methylgag, dexamethasone, cytarabine, methylpredisolone, and mitoxantrone.

22. A method of treating a CD80-positive malignancy in a subject, the method comprising administering to the subject a compound of formula (I) in combination with an anti-CD80 antibody.

23. The method of claim 22, wherein the anti-CD80 antibody is a human, humanized, or chimeric antibody.

24. The method of claim 22, wherein the anti-CD80 antibody binds the same CD80 epitope as the antibody produced by ATCC Deposit No. HB-12119

25. The method of claim 22, wherein the anti-CD80 antibody competes for binding to CD80 with the antibody produced by ATCC Deposit No. HB-12119.

26. The method of claim 22, wherein the anti-CD80 antibody comprises variable regions derived from variable regions of the antibody produced ATCC Deposit No. HB-12119 and human constant regions.

27. The method of claim 22, wherein the anti-CD80 antibody comprises variable regions of the antibody produced by ATCC Deposit No. HB-12119 and human constant regions.

28. The method of claim 23, wherein the human, humanized, or chimeric anti-CD80 antibody comprises human IgG1 or IgG3 constant regions.

29. The method of claim 22, wherein the anti-CD80 antibody induces apoptosis of malignant B cells.

30. The method of claim 22, wherein the anti-CD80 antibody induces cytotoxicity of malignant B cells.

31. The method of claims 22, wherein the anti-CD80 antibody is administered at a dosage of from 0.05 mg/kg body weight to 100 mg/kg body weight.

32. The method of claim 22, wherein the anti-CD80 antibody is administered at a dosage of from 0.5 mg/kg body weight to 10 mg/kg body weight.

33. The method of claim 22, wherein the anti-CD80 antibody is administered parenterally, orally, intraarterially, intramuscularly, rectally, vaginally, or subcutaneously.

34. The method of claim 22, wherein the anti-CD80 antibody is conjugated to a cytotoxin.

35. The method of claim 34, wherein the cytotoxin is a cystotatic agent, an alkylating agent, an anti-metabolite, an anti-proliferative agent, a tubulin binding agent, a hormone, a hormone antagonist, an antibiotic, an enzyme, a radiosensitizing drug, or a cell growth inhibitor.

36. The method of claim 22, wherein the anti-CD80 antibody is radiolabeled.

37. The method of claim 36, wherein the radiolabeled anti-CD80 antibody is ^{131}I-labeled or ^{90}Y-labeled anti-CD80.

38. The method of claim 22, further comprising administering to the subject at least one B cell depleting antibody, wherein the compound of formula (I), anti-CD80 antibody, and the at least one B cell depleting antibody are administered simultaneously or sequentially in any order.

39. The method of claim 38, further comprising administering to the subject at least one chemotherapeutic agent.

40. A method of treating a CD80-positive malignancy in a subject, the method comprising administering to the subject a compound of formula (I) in combination with at least one antibody having B cell depleting activity.

41. The method of claim 40, wherein the B cell depleting antibody binds an antigen selected from the group consisting of CD10, CD19, CD20, CD21, CD22, CD23, CD24, Cd37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD81, CD82, CD83, CDw84, CD85 and CD86 (B7.2)

42. The method of claim 40, wherein the B cell depleting antibody is a monoclonal antibody that binds an antigen selected from the group consisting of CD19, CD20, CD22, CD23, and CD37.

43. The method of claim 40, wherein the B cell depleting antibody is a chimeric, humanized, or human B cell depleting antibody.

44. The method of claim 40, wherein the B cell depleting antibody is an anti-CD20 antibody.

45. The method of claim 44, wherein the anti-CD20 antibody binds same CD20 epitope as the antibody produced by ATCC Deposit No. HB-69119.

46. The method of claim 44, wherein the anti-CD20 antibody competes for binding to CD20 with the antibody produced by ATCC Deposit No. HB-69119.

47. The method of claim 44, wherein the anti-CD20 antibody comprises variable regions derived from variable regions of the antibody produced ATCC Deposit No. HB-69119 and human constant regions.

48. The method of claim 44, wherein the anti-CD20 antibody comprises variable regions of the antibody produced by ATCC Deposit No. HB-69119 and human constant regions.

49. The method of claim 44, wherein the anti-CD20 antibody is rituximab.

50. The method of claim 44, wherein the anti-CD20 antibody induces apoptosis of malignant B cells.

51. The method of claim 44, wherein the anti-CD20 antibody induces cytotoxicity of malignant B cells.

52. The method of claim 44, wherein the anti-CD20 antibody is administered at a dosage of from 0.05 mg/kg body weight to 100 mg/kg body weight.

53. The method of claim 44, wherein the anti-CD20 antibody is administered at a dosage of from 0.5 mg/kg body weight to 10 mg/kg body weight.

54. The method of claim 44, wherein the anti-CD20 antibody is administered parenterally, orally, intraarterially, intramuscularly, rectally, vaginally, or subcutaneously.

55. The method of claim 44, wherein the anti-CD20 antibody is radiolabeled.

56. The method of claim 55, wherein the radiolabeled anti-CD20 antibody is ^{131}I-labeled or ^{90}Y-labeled anti-CD20.

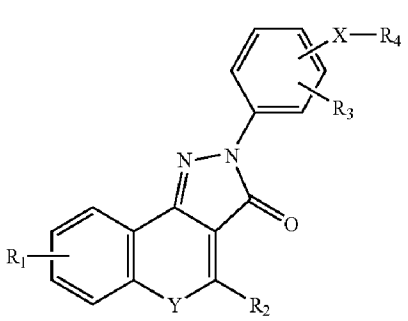
57. The method of claim 44, wherein the anti-CD20 antibody is conjugated to a cytotoxin.

58. The method of claim 57, wherein the cytotoxin is a cystotatic agent, an alkylating agent, an anti-metabolite, an anti-proliferative agent, a tubulin binding agent, a hormone, a hormone antagonist, an antibiotic, an enzyme, a radiosensitizing drug, or a cell growth inhibitor.

59. The method of claim 40, further comprising administering to the subject an anti-CD80 antibody, wherein the compound of formula (I), an antibody having B cell depleting activity, and the anti-CD80 antibody are administered simultaneously or sequentially in any order.

60. The method of claim 38, further comprising administering to the subject at least one chemotherapeutic agent, wherein the compound of formula (I), an antibody having B cell depleting activity, the anti-CD80 antibody, and the at least one chemotherapeutic agent are administered simultaneously or sequentially in any order.

61. A method of inducing apoptosis of CD80-positive malignant cells comprising contacting the cells with an effective amount of a compound of formula (I)



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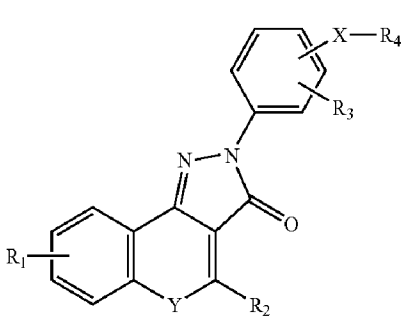
wherein, R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F; R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl; Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl; X represents a bond or a divalent C_1 C_6 alkylene radical; R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -$(Alk)_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7

cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.

62. A method of inducing cytotoxicity of CD80-positive malignant cells comprising contacting the cells with an effective amount of a compound of formula (I)



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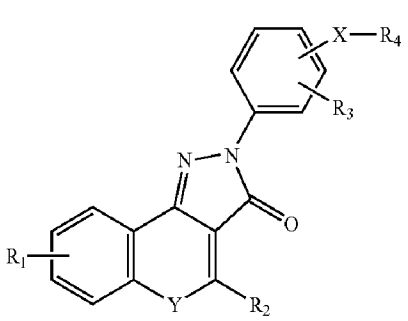
wherein, R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F; R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl; Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl; X represents a bond or a divalent C_1 C_6 alkylene radical; R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -$(Alk)_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7 cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.

63. A method of treating a subject having a malignancy, which comprises a tumor microenvironment of malignant and non-malignant cells, wherein regulatory T cell function contributes to or exacerbates the malignancy, comprising administering to the subject a therapeutically effective amount of a compound of formula (I)



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wherein, R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F; R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl; Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl; X represents a bond or a divalent C_1 C_6 alkylene radical; R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -(Alk)$_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7 cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.

64. The method of claim 63, wherein the malignancy is a hematologic malignancy or a non-hematologic malignancy.

65. The method of claim 64, wherein the malignancy is a hematologic malignancy.

66. The method of claim 65, wherein the hematologic malignancy is lymphoma.

67. The method of claim 66, wherein the lymphoma is B cell lymphoma.

68. The method of claim 67, wherein the B cell lymphoma is Hodgkin's disease.

69. The method of claim 64, wherein the malignancy is a non-hematologic malignancy.

70. The method of claim 69, wherein the non-hematologic malignancy is a cancer of the breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder, bile ducts, small intestine, urinary tract including kidney, bladder and urothelium, female genital tract, cervix, uterus, ovaries, male genital tract, prostate, seminal vesicles, testes, an endocrine gland, thyroid gland, adrenal gland, pituitary gland, skin, bone, soft tissues, blood vessels, brain, nerves, eyes, meninges.

71. The method of claim 63, wherein activation of regulatory T cells, Th2 helper cells, or both regulatory T cells and Th2 cells is inhibited.

72. The method of claim 63, wherein proliferation of regulatory T cells, Th2 helper cells, or both regulatory T cells and Th2 cells is inhibited.

73. The method of claim 63, wherein the compound of formula (I) blocks CD80/CD28 signaling without blocking CD80/CTLA4 signaling.

74. The method of claim 63, wherein production of one or more inflammatory cytokines in the tumor microenvironment is reduced.

75. The method of claim 74, wherein the one or more inflammatory cytokines are selected from the group consisting of interleukin-2, interleukin-4, interleukin-10, interleukin-12, transforming growth factor beta, and interferon-γ.

76. The method of claim 63, wherein non-malignant CD80-expressing cells in the tumor microenvironment are reduced.

77. The method of claim 76, wherein the CD80-expressing cells are antigen presenting cells, myeloid-derived monocytes, or Tie-2-expressing monocytes.

78. The method of claim 63, wherein production of one or more malignant cell survival signals by non-malignant cells of the tumor microenvironment is reduced.

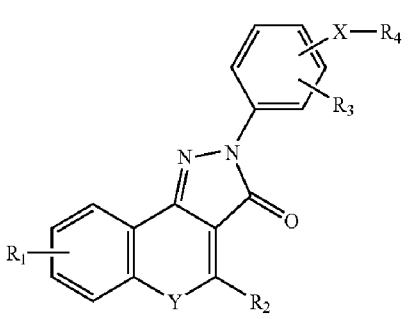
79. The method of claim 78, wherein the one or more survival signals is selected from the group consisting of CD40/CD40L signaling, interleukin-1, or interleukin-6.

80. The method of claim 63, wherein immunity to the malignancy is enhanced in the subject.

81. The method of claim 63, further comprising administering to the subject an anti-cancer agent, wherein the compound of formula (I) and the anti-cancer agent are administered concurrently or consecutively in either order.

82. The method of claim 81, wherein the second therapeutic agent is selected from the group consisting of cytotoxins, radioisotopes, chemotherapeutic agents, immunomodulatory or immunoregulatory agents, anti-angiogenic agents, anti-proliferative agents, pro-apoptotic agents, cytostatic and cytolytic enzymes (*e.g.*, RNAses), enzyme inhibitors (*e.g.*, proteasome inhibitors), and tumor vaccines.

83. A method of inhibiting regulatory T cell and Th2 helper T cell activation in a tumor microenvironment of a subject comprising administering to the subject a therapeutically effective amount of a compound of formula (I)



(I)

wherein, R_1 and R_3 independently represent H; F; Cl; Br; —NO_2; —CN; C_1 C_6 alkyl optionally substituted by F or Cl; or C_1 C_6 alkoxy optionally substituted by F; R_2 represents H, or optionally substituted C_1 C_6 alkyl, C_3 C_7 cycloalkyl or optionally substituted phenyl; Y represents —O—, —S—, N-oxide, or —N(R_5) —wherein R_5 represents H or C_1 C_6 alkyl; X represents a bond or a divalent C_1 C_6 alkylene radical; R_4 represents —C(=O)NR_6R_7, wherein R_6 represents a radical of formula -$(Alk)_b$-Q wherein b is 1 and Alk is an optionally substituted divalent straight chain or branched C_1 C_{12} alkylene, C_2 C_{12} alkenylene or C_2 C_{12} alkynylene radical which may be interrupted by one or more non-adjacent —O—, —S— or —N(R_8) —radicals wherein R_8 represents H or C_1 C_4 alkyl, C_3 C_4 alkenyl, C_3 C_4 alkynyl, or C_3 C_6 cycloalkyl, and Q represents H; —CF_3; —OH; —SH; —NR_8R_8 wherein each R_8 may be the same or different; an ester group; or an optionally substituted phenyl, C_3 C_7 cycloalkyl, C_5 C_7 cycloalkenyl or heterocyclic ring having from 5 to 8 ring atoms; and R_7 represents H or C_1 C_6 alkyl; or when taken together with the atom or atoms to which they are attached R_6 and R_7 form an optionally substituted heterocyclic ring having from 5 to 8 ring atoms.