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(54) **ANTINEOPLASTIC COMBINATIONS**

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(57) **ABSTRACT**

This invention provides the use of a combination of an mTOR inhibitor and an antineoplastic alkylating agent in the treatment of neoplasms.

ANTINEOPLASTIC COMBINATIONS

BACKGROUND OF THE INVENTION

[0001] This application claims priority from copending provisional application Serial No. 60/295,190, filed Jun. 1, 2001, the entire disclosure of which is hereby incorporated by reference.

[0002] This invention relates to the use of combinations of an mTOR inhibitor and an alkylating agent in the treatment of neoplasms.

[0003] Rapamycin is a macrocyclic triene antibiotic produced by *Streptomyces hygroscopicus*, which was found to have antifungal activity, particularly against *Candida albicans*, both in vitro and in vivo [C. Vezina et al., J. Antibiot. 28, 721 (1975); S. N. Sehgal et al., J. Antibiot. 28, 727 (1975); H. A. Baker et al., J. Antibiot. 31, 539 (1978); U.S. Pat. Nos. 3,929,992; and 3,993,749]. Additionally, rapamycin alone (U.S. Pat. No. 4,885,171) or in combination with picibanil (U.S. Pat. No. 4,401,653) has been shown to have antitumor activity.

[0004] The immunosuppressive effects of rapamycin have been disclosed in FASEB a* 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); R. Y. Calne et al., Lancet 1183 (1978); and U.S. Pat. No. 5,100,899]. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

[0005] Rapamycin is also useful in preventing or treating systemic lupus erythematosus [U.S. Pat. No. 5,078,999], pulmonary inflammation [U.S. Pat. No. 5,080,899], insulin dependent diabetes mellitus [U.S. Pat. No. 5,321,009], skin disorders, such as psoriasis [U.S. Pat. No. 5,286,730], bowel disorders [U.S. Pat. No. 5,286,731], smooth muscle cell proliferation and intimal thickening following vascular injury [U.S. Pat. Nos. 5,288,711 and 5,516,781], adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1], ocular inflammation [U.S. Pat. No. 5,387,589], malignant carcinomas [U.S. Pat. No. 5,206,018], cardiac inflammatory disease [U.S. Pat. No. 5,496,832], and anemia [U.S. Pat. No. 5,561,138].

[0006] Rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) is ester of rapamycin which has demonstrated significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of hydroxyesters of rapamycin, including CCI-779, are disclosed in U.S. Pat. No. 5,362,718.

[0007] CCI-779 exhibits cytostatic, as opposed to cytotoxic properties, and may delay the time to progression of tumors or time to tumor recurrence. CCI-779 is considered to have a mechanism of action that is similar to that of sirolimus. CCI-779 binds to and forms a complex with the cytoplasmic protein FKBP, which inhibits an enzyme, mTOR (mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]). Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S. The mechanism of action of CCI-779 that results in the G1 S phase block is novel for an anticancer drug.

[0008] In vitro, CCI-779 has been shown to inhibit the growth of a number of histologically diverse tumor cells. Central nervous system (CNS) cancer, leukemia (T-cell), breast cancer, prostate cancer, and melanoma lines were among the most sensitive to CCI-779. The compound arrested cells in the G1 phase of the cell cycle.

[0009] In vivo studies in nude mice have demonstrated that CCI-779 has activity against human tumor xenografts of diverse histological types. Gliomas were particularly sensitive to CCI-779 and the compound was active in an orthotopic glioma model in nude mice. Growth factor (platelet-derived)-induced stimulation of a human glioblastoma cell line in vitro was markedly suppressed by CCI-779. The growth of several human pancreatic tumors in nude mice as well as one of two breast cancer lines studied in vivo also was inhibited by CCI-779.

DESCRIPTION OF THE INVENTION

[0010] This invention provides the use of combinations of an mTOR inhibitor and an antineoplastic alkylating agent as antineoplastic combination chemotherapy. In particular, these combinations are useful in the treatment of renal cancer, soft tissue cancer, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small lung cell cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. This invention also provides combinations of an mTOR inhibitor and an antineoplastic alkylating agent for use as antineoplastic combination chemotherapy, in which the dosage of either the mTOR inhibitor or the antineoplastic alkylating agent or both are used in subtherapeutically effective dosages.

[0011] As used in accordance with this invention, the term "treatment" means treating a mammal having a neoplastic disease by providing said mammal an effective amount of a combination of an mTOR inhibitor and an antineoplastic alkylating agent with the purpose of inhibiting growth of the neoplasm in such mammal, eradication of the neoplasm, or palliation of the mammal.

[0012] As used in accordance with this invention, the term "providing," with respect to providing the combination, means either directly administering the combination, or administering a prodrug, derivative, or analog of one or both of the components of the combination which will form an effective amount of the combination within the body.

[0013] mTOR is the mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]. Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated

cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S.

[0014] mTOR regulates the activity of at least two proteins involved in the translation of specific cell cycle regulatory proteins (Burnett, P. E., PNAS 95: 1432 (1998) and Isotani, S., J. Biol. Chem. 274: 33493 (1999)). One of these proteins p70s6 kinase is phosphorylated by mTOR on serine 389 as well as threonine 412. This phosphorylation can be observed in growth factor treated cells by Western blotting of whole cell extracts of these cells with antibody specific for the phosphoserine 389 residue.

[0015] As used in accordance with this invention, an "mTOR inhibitor" means a compound or ligand which inhibits cell replication by blocking progression of the cell cycle from G1 to S by inhibiting the phosphorylation of serine 389 of p70s6 kinase by mTOR.

[0016] The following standard pharmacological test procedure can be used to determine whether a compound is an mTOR inhibitor, as defined herein. Treatment of growth factor stimulated cells with an mTOR inhibitor like rapamycin completely blocks phosphorylation of serine 389 as evidenced by Western blot and as such constitutes a good assay for mTOR inhibition. Thus whole cell lysates from cells stimulated by a growth factor (eg. IGF1) in culture in the presence of an mTOR inhibitor should fail to show a band on an acrylamide gel capable of being labeled with an antibody specific for serine 389 of p70s6K.

Materials:

NuPAGE LDS Sample Buffer	(Novex Cat # NP0007)
NuPAGE Sample Reducing Agent	(Novex Cat # NP0004)
NuPAGE 4–12% Bis-Tris Gel	(Novex Cat # NP0321)
NuPAGE MOPS SDS Running Buffer	(Novex Cat # NP0001)
Nitrocellulose	(Novex Cat # LC2001)
NuPAGE Transfer Buffer	(Novex Cat # NP0006)
Hyperfilm ECL	(Amersham Cat # RPN3114H)
ECL Western Blotting Detection Reagent	(Amersham Cat # RPN2134)
Primary antibody: Phospho-p70 S6 Kinase (Thr389)	(Cell Signaling Cat # 9205)
Secondary antibody: Goat anti-rabbit IgG-HRP conjugate	(Santa Cruz Cat # sc-2004)

[0017] Methods

[0018] A. Preparation of Cell Lysates

[0019] Cell lines were grown in optimal basal medium supplemented with 10% fetal bovine serum and penicillin/treptomycin. For phosphorylation studies, cells were subcultured in 6-well plates. After the cells have completely attached, they were either serum-starved. Treatment with mTOR inhibitors ranged from 2 to 16 hours. After drug treatment, the cells were rinsed once with PBS (phosphate buffered saline without Mg++ and Ca++) and then lysed in 150-200 μl NuPAGE LDS sample buffer per well. The lysates were briefly sonicated and then centrifuged for 15 minutes at 14000 rpm. Lysates were stored at minus -80° C. until use.

[0020] The test procedure can also be run by incubating the cells in growth medium overnigh, after they have completely attached. The results under both sets of conditions should be the same for an mTOR inhibitor.

[0021] B. Western Blot Analysis

[0022] 1) Prepare total protein samples by placing 22.5 μl of lysate per tube and then add 2.5 μl NuPAGE sample reducing agent. Heat samples at 70° C. for 10 minutes. Electrophoresed using NuPAGE gels and NuPAGE SDS buffers.

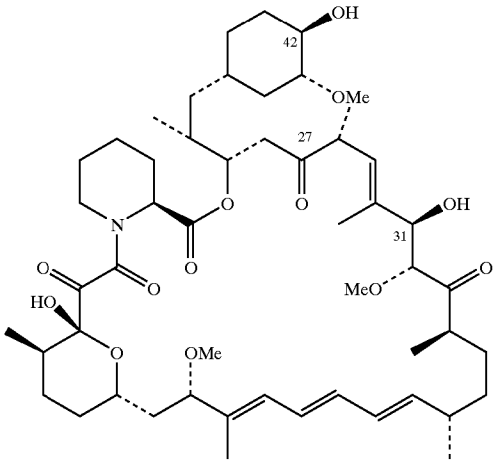
[0023] 2) Transfer the gel to a nitrocellulose membrane with NuPAGE transfer buffer. The membrane are blocked for 1 hour with blocking buffer (Tris buffered saline with 0.1%-Tween and 5% nonfat-milk). Rinse membranes 2× with washing buffer (Tris buffered saline with 0.1%-Tween).

[0024] 3) Blots/membrane are incubated with the P-p70 S6K (T389) primary antibody (1:1000) in blocking buffer overnight at 4° C. in a rotating platform.

[0025] 4) Blots are rinsed 3× for 10 minutes each with washing buffer, and incubated with secondary antibody (1:2000) in blocking buffer for 1 hour at room temperature.

[0026] 5) After the secondary antibody binding, blots are washed 3× for 10 minutes each with washing buffer, and 2× for 1 minute each with Tris-buffered saline, followed by chemiluminescent (ECL) detection and then exposed to chemiluminescence films.

[0027] As used in accordance with this invention, the term "a rapamycin" defines a class of immunosuppressive compounds which contain the basic rapamycin nucleus (shown below). The rapamycins of this invention include compounds which may be chemically or biologically modified as derivatives of the rapamycin nucleus, while still retaining immunosuppressive properties. Accordingly, the term "a rapamycin" includes esters, ethers, oximes, hydrazones, and hydroxylamines of rapamycin, as well as rapamycins in which functional groups on the rapamycin nucleus have been modified, for example through reduction or oxidation. The term "a rapamycin" also includes pharmaceutically acceptable salts of rapamycins, which are capable of forming such salts, either by virtue of containing an acidic or basic moiety.



RAPAMYCIN

[0028] It is preferred that the esters and ethers of rapamycin are of the hydroxyl groups at the 42- and/or 31-positions of the rapamycin nucleus, esters and ethers of a hydroxyl group at the 27-position (following chemical reduction of the 27-ketone), and that the oximes, hydrazones, and hydroxylamines are of a ketone at the 42-position (following oxidation of the 42-hydroxyl group) and of 27-ketone of the rapamycin nucleus.

[0029] Preferred 42- and/or 31-esters and ethers of rapamycin are disclosed in the following patents, which are all hereby incorporated by reference: alkyl esters (U.S. Pat. No. 4,316,885); aminoalkyl esters (U.S. Pat. No. 4,650,803); fluorinated esters (U.S. Pat. No. 5,100,883); amide esters (U.S. Pat. No. 5,118,677); carbamate esters (U.S. Pat. No. 5,118,678); silyl ethers (U.S. Pat. No. 5,120,842); aminoesters (U.S. Pat. No. 5,130,307); acetals (U.S. Pat. No. 5,51,413); aminodiesters (U.S. Pat. No. 5,162,333); sulfonate and sulfate esters (U.S. Pat. No. 5,177,203); esters (U.S. Pat. No. 5,221,670); alkoxyesters (U.S. Pat. No. 5,233,036); O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Pat. No. 5,258,389); carbonate esters (U.S. Pat. No. 5,260,300); arylcarbonyl and alkoxycarbonyl carbamates (U.S. Pat. No. 5,262,423); carbamates (U.S. Pat. No. 5,302,584); hydroxyesters (U.S. Pat. No. 5,362,718); hindered esters (U.S. Pat. No. 5,385,908); heterocyclic esters (U.S. Pat. No. 5,385,909); gem-disubstituted esters (U.S. Pat. No. 5,385,910); amino alkanoic esters (U.S. Pat. No. 5,389,639); phosphorylcarbamate esters (U.S. Pat. No. 5,391,730); carbamate esters (U.S. Pat. No. 5,411,967); carbamate esters (U.S. Pat. No. 5,434,260); amidino carbamate esters (U.S. Pat. No. 5,463,048); carbamate esters (U.S. Pat. No. 5,480,988); carbamate esters (U.S. Pat. No. 5,480,989); carbamate esters (U.S. Pat. No. 5,489,680); hindered N-oxide esters (U.S. Pat. No. 5,491,231); biotin esters (U.S. Pat. No. 5,504,091); O-alkyl ethers (U.S. Pat. No. 5,665,772); and PEG esters of rapamycin (U.S. Pat. No. 5,780,462). The preparation of these esters and ethers are disclosed in the patents listed above.

[0030] Preferred 27-esters and ethers of rapamycin are disclosed in U.S. Pat. No. 5,256,790, which is hereby incorporated by reference. The preparation of these esters and ethers are disclosed in the patents listed above.

[0031] Preferred oximes, hydrazones, and hydroxylamines of rapamycin are disclosed in U.S. Pat. Nos. 5,373,014, 5,378,836, 5,023,264, and 5,563,145, which are hereby incorporated by reference. The preparation of these oximes, hydrazones, and hydroxylamines are disclosed in the above listed patents. The preparation of 42-oxorapamycin is disclosed in U.S. Pat. No. 5,023,263, which is hereby incorporated by reference.

[0032] Particularly preferred rapamycins include rapamycin [U.S. Pat. No. 3,929,992], CCI-779 [rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid; U.S. Pat. No. 5,362,718], and 42-O-(2-hydroxy)ethyl rapamycin [U.S. Pat. No. 5,665,772].

[0033] When applicable, pharmaceutically acceptable salts of the rapamycin can be formed from organic and inorganic acids, for example, acetic, propionic, lactic, citric, tartaric, succinic, fumaric, maleic, malonic, mandelic, malic, phthalic, hydrochloric, hydrobromic, phosphoric, nitric, sulfuric, methanesulfonic, napthalenesulfonic, benzenesulfonic, toluenesulfonic, camphorsulfonic, and similarly known acceptable aids when the rapamycin contains a suitable basic moiety. Salts may also be formed from organic and inorganic bases, such as alkali metal salts (for example, sodium, lithium, or potassium) alkaline earth metal salts, ammonium salts, alkylammonium salts containing 1-6 carbon atoms or dialkylammonium salts containing 1-6 carbon atoms in each alkyl group, and trialkylammonium salts containing 1-6 carbon atoms in each alkyl group, when the rapamycin contains a suitable acidic moiety.

[0034] It is preferred that the mTOR inhibitor used in the antineoplastic combinations of this invention is a rapamycin, and more preferred that the mTOR inhibitor is rapamycin, CCI-779, or 42-O-(2-hydroxy)ethyl rapamycin.

[0035] As described herein, CCI-779 was evaluated as a representative mTOR inhibitor in the mTOR inhibitor plus antimetabolite combinations of this invention.

[0036] The preparation of CCI-779 is described in U.S. Pat. No. 5,362,718, which is hereby incorporated by reference. When CCI-779 is used as an antineoplastic agent, it is projected that initial i.v. infusion dosages will be between about 0.1 and 100 mg/m^2 when administered on a daily dosage regimen (daily for 5 days, every 2-3 weeks), and between about 0.1 and 1000 mg/m^2 when administered on a once weekly dosage regimen. Oral or intravenous infusion are the preferred routes of administration, with intravenous being more preferred.

[0037] As used in accordance with this invention, the term "antineoplastic alkylating agent" means a substance which reacts with (or "alkylates") many electron-rich atoms in cells to form covalent bonds. The most important reactions with regard to their antitumor activities are reactions with DNA bases. Some alkylating agents are monofunctional and react with only one strand of DNA. Others are bifunctional and react with an atom on each of the two strands of DNA to produce a "cross-link" that covalently links the two strands of the DNA double helix. Unless repaired, this lesion will prevent the cell from replicating effectively. The lethality of

the monofunctional alkylating agents results from the recognition of the DNA lesion by the cell and the response of the cell to that lesion. (Colvin O M. Antitumor Alkylating Agents. In Cancer Principles & Practice of Oncology 6th Edition. ed. DeVita V T, Hellman S, Rosenberg S A. Lippincoft Williams & Wilkins. Philadelphia 2001. p. 363.)

[0038] Antineoplastic alkylating agents are roughly classified, according to their structure or reactive moiety, into several categories which include nitrogen mustards, such as mustargen, cyclophosphamide, ifosfamide, melphalan, and chlorambucil; azidines and epoxides, such as thiotepa, mitomycin C, dianhydrogalactitol, and dibromodulcitol; alkyl sulfonates, such as busulfan; nitrosoureas, such as bischloroethylnitrosourea (BCNU), cyclohexyl-chloroethylnitrosourea (CCNU), and methylcyclohexylchloroethylnitrosourea (MeCCNU); hydrazine and triazine derivatives, such as procarbazine, dacarbazine, and temozolomide; and platinum compounds. Platinum compounds are platinum containing agents that react preferentially at the N7 position of guanine and adenine residues to form a variety of monofunctional and bifunctional adducts. (Johnson S W, Stevenson J P, O'Dwyer P J. Cisplatin and Its Analogues. In Cancer Principles & Practice of Oncology 6th Edition. ed. DeVita V T, Hellman S, Rosenberg S A. Lippincott Williams & Wilkins. Philadelphia 2001. p. 378.) These compounds include cisplatin, carboplatin, platinum IV compounds, and multinuclear platinum complexes.

[0039] The following are representative examples of alkylating agents of this invention.

[0040] Meclorethamine is commercially available as an injectable (MUSTARGEN).

[0041] Cyclophosphamide is commercially available as an injectable (cyclophosphamide, lyophilized CYTOXAN, or NEOSAR) and in oral tablets (cyclophosphamide or CYTOXAN).

[0042] Ifosfamide is commercially available as an injectable (IFEX).

[0043] Melphalan is commercially available as an injectable (ALKERAN) and in oral tablets (ALKERAN).

[0044] Chlorambucil is commercially available in oral tablets (LEUKERAN).

[0045] Thiotepa is commercially available as an injectable (thiotepa or THIOPLEX).

[0046] Mitomycin is commercially available as an injectable (mitomycin or MUTAMYCIN).

[0047] Busulfan is commercially available as an injectable (BUSULFEX) and in oral tablets (MYLERAN).

[0048] Lomustine (CCNU) is commercially available in oral capsules (CEENU).

[0049] Carmustine (BCNU) is commercially available as an intracranial implant (GLIADEL) and as an injectable (BICNU).

[0050] Procarbazine is commercially available in oral capsules (MATULANE).

[0051] Temozolomide is commercially available in oral capsules (TEMODAR).

[0052] Cisplatin is commercially available as an injectable (cisplatin, PLATINOL, or PLATINOL-AQ).

[0053] Carboplatin is commercially available as an injectable (PARAPLATIN).

[0054] The following table briefly summarizes some of the recommended dosages for the antineoplastic alkylating agents listed above.

TABLE 1

Recommended Dosages of Antineoplastic Alkylating Agents

Drug	Dosage	Regimen
Mustargen	0.4 mg/kg	each course given as a singe dose or in divided doses of 0.1 to 0.2 mg/kg/day.
Cyclophosphamide	40–50 mg/kg i.v.	in divided doses over a period of 2–5 days
	10–15 mg/kg i.v.	every 7–10 days
	3–5 mg/kg i.v.	twice weekly
	1–5 mg/kg oral	daily
Ifosfamide	1.2 g/m^2 i.v.	daily for 5 consecutive days; repeated every 3 weeks or after recovery from hematologic toxicity.
Melphalan	6 mg orally	daily for 2–3 weeks followed by 4 weeks rest, then 2 mg daily maintenance dosage
	10 mg orally	daily for 7–10 days followed by 2 mg daily maintenance after white blood cell count has recovered.
	0.15 mg/kg orally	daily for 7 days, followed by a rest period of at least 14 days, then 0.005 mg/kg daily maintenance.
	16 mg/m^2 i.v.	single infusion over 15–20 minutes every 2 weeks for 4 doses, followed by a rest period, then administered at 4 week intervals for maintenance.
Chlorambucil	0.1–0.2 mg/kg orally	daily for 3–6 weeks
Thiotepa	0.3–0.4 mg/kg i.v.	every 1–4 weeks
Mitomycin	20 mg/m^2 i.v.	every 6–8 weeks
Busulfan	1.8 mg/m^2 orally	daily

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