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(54) Title: SMALL MOLECULE ENHANCER FOR DENDRITIC CELL CANCER VACCINES

(57) Abstract: Disclosed is a method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases. Also disclosed is a method of (a) increasing antitumor immunity, (b) stimulating or enhancing an immune response, (c) treating a condition characterized by abnormal cell proliferation, (d) increasing cytokine and/or chemokine production, or (e) stimulating or enhancing production of T-cells, in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases. For example, the compound that inhibits a plurality of mammalian DASH serine proteases may be *t*-butylGly-boroPro.

Small Molecule Enhancer for Dendritic Cell Cancer Vaccines

Related Applications

This application claims the benefit of priority to United States Provisional Patent Application serial number 61/562,497, filed November 22, 2011.

Background of the Invention

Cancer is America's second leading cause of death. Approved anticancer agents, both chemotherapeutic and targeted agents, are limited by toxicity and are ultimately ineffective against solid tumors, e.g.: lung, colorectal, breast, pancreatic, and prostate cancers, which account for more than 85% of cancer deaths. To kill tumors using the body's immune system, the failure of which has allowed the cancer to emerge, has long been the goal of cancer research. Val-boroPro, also known as PT-100 or talabostat, is a dipeptide boronic acid that showed remarkable efficacy in shrinking tumors in mice through immune activation. However, in Fast Track Phase III clinical trials, it did not meet its objectives, due to dose-limiting toxicity.

The US Food and Drug Administration approved the first cancer vaccine, Provenge for prostate cancer, on April 29, 2010. Provenge is a dendritic cell therapy (DCT); one of several exciting new immunotherapies sometimes called "cancer vaccines". By supercharging the immune system, such vaccines can, in principle, find and remove the very last cancer cell, no matter where it hides, thus precluding mere remission after a course of treatment. Although the concept is now proven, cancer vaccines, including DCTs other than Provenge, have failed to achieve the desired efficacy in clinical trials, indicating the need to add immune stimulators, or *adjuvants*. However, less toxic adjuvants are needed to develop this approach clinically.

Summary of the Invention

One aspect of the present invention relates to a method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

Another aspect of the invention relates to a method of increasing antitumor immunity in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

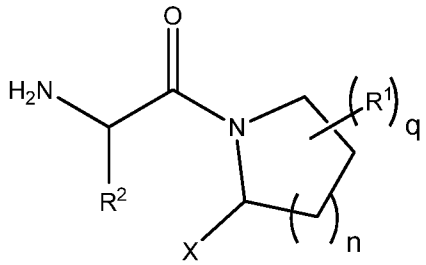
Another aspect of the invention relates to a method of stimulating or enhancing an immune response in a mammal, comprising administering to a mammal in need of an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

Yet another aspect of the invention relates to a method of treating a condition characterized by abnormal cell proliferation, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

Another aspect of the invention relates to a method of increasing cytokine and chemokine production in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

Another aspect of the invention relates to a method of stimulating or enhancing production of T-cells in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases, wherein said T cells recognize an antigen on a malignant cell.

In certain embodiments, the invention relates to any one of the methods described above, wherein the compound is represented by Formula I:



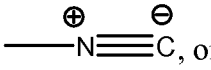
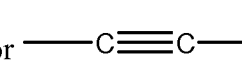
Formula I

wherein:

X is $B(Y^1)(Y^2)$ or CN;

Y^1 and Y^2 are independently OH, or together with the boron atom to which they are attached represent a group that is hydrolysable to a boronic acid, or together with the boron atom to which they are attached form a 5- to 8-membered ring that is hydrolysable to a boronic acid;

R^1 is selected from the group consisting of halogen, lower alkyl, lower alkenyl, lower alkynyl, carbonyl, carboxyl, ester, formate, ketone, thiocarbonyl, thioester, thioacetate, thioformate, amino, acylamino, amido, nitro, sulfate, sulfonate,

sulfonamido, -$(CH_2)_m$-R_7, -$(CH_2)_m$-OH, -$(CH_2)_m$-O-lower alkyl, -$(CH_2)_m$-O-lower alkenyl, -$(CH_2)_n$-O-$(CH_2)_m$-R_7, -$(CH_2)_m$-SH, -$(CH_2)_m$-S-lower alkyl, -$(CH_2)_m$-S-lower alkenyl, or -$(CH_2)_n$-S-$(CH_2)_m$-R_7, azido, cyano, isocyanato, thiocyanato, isothiocyanato, cyanato,  , or  ;

R_7 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle;

R_8 independently represents hydrogen, -CH_3, or -$(CH_2)_n$-CH_3;

m is 0, 1, 2, 3, 4, 5, or 6;

R^2 is a hydrophobic group selected from the group consisting of *n*-propyl, C_4-C_8 alkyl, C_2-C_8alkenyl, C_2-C_8alkynyl, C_3-C_8cycloalkyl, C_2-C_7heterocyclyl, aryl, heteroaryl, aralkyl, heteroaralkyl, and side chains of naturally occurring hydrophobic amino acids;

n is 0, 1, or 2; and

q is 0, 1, 2, 3, or 4.

In certain embodiments, the invention relates to any one of the methods described above, wherein the compound is *t*-butylGly-boroPro.

Brief Description of the Drawings

Figure 1 shows that Ala-boroPro (A-bP) and Val-boroPro (V-bP) both inhibit prolyl oligoproteases, but Ala-boroPro does not stimulate the immune system. Val-boroPro is a potent immune stimulator.

Figure 2 shows that PT-100 produces complete regression of early stage tumors but not of established tumors.

Figure 3 shows that PT-100 + Dendritic Cell (DC) vaccine produces regression of established tumors.

Figure 4 shows that Val-boroPro (2054 in the figure), but not Ala-boroPro (2054 in the figure), stimulates G-CSF and CXCL1/KC in BALB/c mice. G-CSF and CXL1/KC are markers of anti-cancer immune enhancing activity. The experimental setup and results are discussed in Example 3.

Figure 5 shows that Ari-4175 (4175-2 in the figure) is a very potent inducer of cytokines *in vivo*. The experimental setup and results are discussed in Example 3.

Figure 6 shows that ARI-4175 (4175-2) is much more potent than PT-100 (2054) at inducing G-CSF and CXCL1 cytokines, which are markers of anti-cancer immune-enhancing activity. The experimental setup and results are discussed in Example 3.

Figure 7 shows that ARI-4175 establishes immunity to tumor rechallenge in the M3-9-M RMS model. The experimental setup and results are discussed in Example 4.

Figures 8 A and **B** show that ARI-4175 increases potency of a DC vaccine in a rhabdomyosarcoma (RMS) model to inhibit growth of established RMS and increase mouse survival. C57BL/6 mice were injected intramuscularly with RMS cells on day 0. Mice received a single subcutaneous vaccination on day 10 followed by daily gavage of 10 mg/kg (5 days) and 5 mg/kg (10 days) ARI-4175, 1 mg/kg PT-100 (15 days), or vehicle as described in Example 1.

Figure 9 shows tumor growth in individual mice in the experiment described in Experiment 1.

Figure 10 shows that ARI-4175 (4175) does not exhibit significant *in vitro* activity against colorectal cancer cell lines. There is also no activity when 4175 is combined with cetuximab (CTX). This is expected, since the antitumor effects of ARI-4175 and cetuximab are thought to be mediated through antibody-dependent cell-mediated cytotoxicity (ADCC).

Figure 11 shows that CD16 expression is upregulated on NK cells taken from nude mice treated with ARI-4175.

Figure 12 shows that LAMP-1 (CD107) expression is upregulated on NK cells taken from nude mice treated with ARI-4175.

Figures 13 A and **B** show the *in vivo* inhibition by ARI-4175 of colon cancer xenografts. **A** shows the inhibition of DLD1 xenografts by ARI-4175 alone or in combination with cetuximab (CTX). **B** shows the inhibition of HCT-116 by ARI-4175 alone or in combination with cetuximab (CTX).

Figure 14 shows that cytotoxicity of splenocytes from C57BI/6 non-tumor-bearing mice treated with ARI-4175 and/or cetuximab (CTX) is enhanced against HCT116 tumor cells.

Figure 15 shows that ARI-4175 induces CD69 on human NK cells. The results shown are with cultured human PBLs from two healthy donors after a 1 day incubation.

Figure 16 shows that ARI-4175 is as effective as PT-100 but less toxic at a high dose.

Figures 17 A and **B** show that as adjuvant to tumor-primed T cell transfer, ARI-4175 induces tumor regression in late treatment RMS M3-9-M model.

Figures 18 A and **B** show that combination treatment with ARI-4175 and adoptive T cell transfer in Rag1-/- recipients significantly reduces RMS M3-9-M volume. (A)

Female Rag1$^{-/-}$ mice received naïve or RMS-primed T cells one day after tumor challenge. (B) By day 10, ARI-4175 treated mice had significantly smaller tumors than saline treated mice (naïve: n=5, p=0.0159; primed: n=5, p=0.0079).

Rag1$^{-/-}$ mice treated with tumor-primed T cells in combination with ARI-4175 had the smallest tumors overall.

Figure 19 ARI-4175 is a potent adjuvant to DC vaccination as evidenced by significantly improved survival and reduced RMS M3-9-M volume.

Figure 20 shows the results of a maximum tolerated dose (MTD) study using SD rats with the data taken 30 minutes post-dose.

Figure 21 shows a dose-plasma response curve for PT-100 and ARI-4175.

Figure 22 shows pharmacokinetic data for the open vs. closed forms of ARI-4175. Under acidic conditions, ARI-4175 exists in an open, or linear, form; under neutral or basic conditions, a closed, cyclized form is highly favored.

Figure 23 shows tumor growth in individual mice in the RMS model.

Detailed Description of the Invention

One aspect of the present invention relates to a method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

Another aspect of the present invention relates to a method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases, wherein the compound is not Val-boroPro.

Another aspect of the present invention relates to any one of the foregoing methods, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

Another aspect of the present invention relates to any one of the foregoing methods, wherein the cancer is selected from the group consisting of basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain cancer, breast cancer, cervical cancer, choriocarcinoma, CNS cancer, colon and rectum cancer, connective tissue cancer, cancer of the digestive system, endometrial cancer, esophageal cancer, eye cancer, cancer of the head and neck, gastric cancer, intra-epithelial neoplasm, kidney cancer, larynx cancer, leukemia, acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, chronic lymphoid leukemia, liver cancer, small cell lung cancer, non-small cell lung cancer,

lymphoma, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, melanoma, myeloma, neuroblastoma, oral cavity cancer, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, rectal cancer, renal cancer, cancer of the respiratory system, sarcoma, skin cancer, stomach cancer, testicular cancer, thyroid cancer, uterine cancer, and cancer of the urinary system.

In other embodiments, the cancer is selected from the group consisting of prostate cancer, colorectal cancer, multiple myeloma, and non-small cell lung cancer.

In certain other embodiments, the cancer is selected from lung cancer, colorectal cancer, breast cancer, pancreatic cancer and prostate cancer.

In one embodiment, the cancer is lung cancer.

In another embodiment, the cancer is non-small cell lung cancer.

In yet another embodiment, the cancer is colorectal cancer.

In certain embodiments, the cancer is breast cancer.

In certain other embodiments, the cancer is pancreatic cancer.

In another embodiment, the cancer is prostate cancer.

In certain embodiments, the cancer is metastatic.

Another aspect of the invention relates to any one of the methods described above, further comprising co-administering to the mammal a therapeutically effective amount of tumor-primed T-cells.

In certain embodiments, the tumor-primed T-cells are administered prior to the administration of the compound.

In certain embodiments, the tumor-primed T-cells are administered subsequent to the administration of the compound.

In certain embodiments, the tumor-primed T-cells are administered concurrently with administration of the compound.

Another aspect of the invention relates to any one of the methods described above, further comprising co-administering to the mammal a therapeutically effective amount of an orally active tumor antigen.

Yet another aspect of the invention relates to any one of the methods described above, further comprising co-administering to the mammal a therapeutically effective amount of a dendritic cell vaccine.

Still another aspect of the invention relates to any one of the methods described above, further comprising administration of an adjuvant.

Another aspect of the present invention relates to any one of the aforementioned embodiments, further comprising treating the mammal with a second therapy selected from the group consisting of surgery, radiation and chemotherapy.

In one embodiment, the second therapy is surgery.

In another embodiment, the second therapy is radiation.

In yet another embodiment, the second therapy is chemotherapy.

In certain embodiments, the chemotherapy is selected from the group consisting of of ipilimumab, vemurafenib, GDC-0879, PLX-4720, aldesleukin, asparaginase, bleomycin sulfate, carboplatin, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin hydrochloride, docetaxel, doxorubicin, doxorubicin hydrochloride, epirubicin hydrochloride, etoposide, etoposide phosphate, floxuridine, fludarabine, fluorouracil, gemcitabine, gemcitabine hydrochloride, hydroxyurea, idarubicin hydrochloride, ifosfamide, interferons, interferon-α2a, interferon-α2b, interferon-αn3, interferon-α1b, interleukins, irinotecan, mechlorethamine hydrochloride, melphalan, mercatopurine, methotrexate, methotrexate sodium, mitomycin, mitoxantrone, paclitaxel, pegaspargase, pentostatin, prednisone, profimer sodium, procabazine hydrochloride, taxol, taxotere, teniposide, topotecan hydrochloride, vinblastine sulfate, vincristine sulfate and vinorelbine tartrate.

In certain embodiments the chemotherapy is selected from the group consisting of bleomycin sulfate, carboplatin, cisplatin, docetaxel, doxorubicin, doxorubicin hydrochloride, fluorouracil, gemcitabine, gemcitabine hydrochloride, methotrexate, methotrexate sodium, paclitaxel, taxol, taxotere, vinblastine sulfate and vincristine sulfate.

In certain embodiments, the chemotherapy is a dipeptidylpeptidase IV inhibitor.

In certain other embodiments, the chemotherapy is a FAP-activated chemotherapeutic, a FAP-activated dipeptidylpeptidase IV inhibitor, or a FAP-activated proteasome inhibitor.

In still other embodiments, the chemotherapy is a FAP-activated proteasome inhibitor.

In certain embodiments, the chemotherapy is an antibody.

In certain other embodiments, the antibody is selected from the group consisting of trastuzamab, cetuximab, bevacizumab, and rituximab.

One aspect of the present invention relates to a method of increasing antitumor immunity in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

In certain embodiments, the compound is not Val-boroPro.

In certain other embodiments, the compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

In yet other embodiments, the antitumor immunity is increased for tumors selected from the group consisting of lung tumors, lymphomas, breast tumors, colorectal tumors, thyroid tumors, uterine tumors, pancreatic tumors, prostate tumors, skin tumors, kidney tumors, liver tumors and brain tumors.

In other embodiments, the antitumor immunity is increased for tumors selected from the group consisting of lung tumors, breast tumors, colorectal tumors, pancreatic tumors and prostate tumors.

In certain other embodiments, the antitumor immunity comprises antibody-dependent cell-mediated cytotoxicity.

Another aspect of the invention relates to a method of stimulating or enhancing an immune response in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

In certain embodiments, the compound is not Val-boroPro.

In certain other embodiments, the compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

In still other embodiments, the immune response is stimulated.

In still yet further embodiments, the immune response is enhanced.

In certain embodiments, the immune response comprises antibody-dependent cell-mediated cytotoxicity.

In certain other embodiments, the mammal has cancer or is at risk of developing cancer.

In still other embodiments, the mammal is in remission of cancer.

In still yet further embodiments, the mammal has a refractory or resistant cancer.

Another aspect of the invention relates to a method of treating a condition characterized by abnormal cell proliferation, comprising administering to a mammal in

need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

In certain embodiments, the compound is not Val-boroPro.

In certain embodiments, the compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

In certain embodiments, the abnormal cell proliferation is cancer, a blood vessel proliferative disorder or a fibrotic disorder.

In certain embodiments, the abnormal cell proliferation is abnormal angiogenesis.

Another aspect of the invention relates to a method of increasing cytokine and/or chemokine production in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

In certain embodiments, the compound is not Val-boroPro.

In certain other embodiments, the compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

Another aspect of the invention relates to a method of stimulating or enhancing production of T-cells in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases, wherein said T-cells recognize an antigen on a malignant cell.

In certain embodiments, the compound is not Val-boroPro.

In certain other embodiments, the compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

In certain other embodiments, the production of T-cells is stimulated.

In yet other embodiments, the production of T-cells is enhanced.

In still yet other embodiments, the malignant cell is a carcinoma, sarcoma, leukemia, lymphoma or myeloma.

In certain embodiments, the mammal is a primate, canine, equine, feline or bovine.

In certain other embodiments, the mammal is a human.

In certain embodiments, the compound is administered orally or parenterally.

In certain other embodiments, the compound is administered parenterally.

In yet other embodiments, the compound is administered orally.

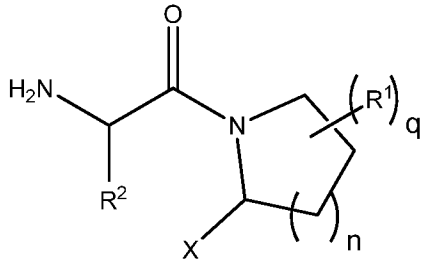
In certain embodiments, the compound is administered in a solid dosage form.

In certain other embodiments, the solid dosage form is a tablet, capsule or pill.

In yet other embodiments, the solid dosage form is a tablet.

In certain embodiments, the compound is administered in an amount sufficient to stimulate the immune system without dose limiting toxicity.

In certain embodiments, the invention relates to any one of the methods described above, wherein the compound is represented by Formula I:

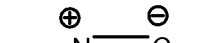


Formula I

wherein:

X is $B(Y^1)(Y^2)$ or CN;

Y^1 and Y^2 are independently OH, or together with the boron atom to which they are attached represent a group that is hydrolysable to a boronic acid, or together with the boron atom to which they are attached form a 5- to 8-membered ring that is hydrolysable to a boronic acid;

R^1 is selected from the group consisting of halogen, lower alkyl, lower alkenyl, lower alkynyl, carbonyl, carboxyl, ester, formate, ketone, thiocarbonyl, thioester, thioacetate, thioformate, amino, acylamino, amido, nitro, sulfate, sulfonate, sulfonamido, $-(CH_2)_m-R_7$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_7$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, or $-(CH_2)_n-S-(CH_2)_m-R_7$, azido, cyano, isocyanato, thiocyanato, isothiocyanato, cyanato,  , or ;

R_7 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle;

R_8 independently represents hydrogen, $-CH_3$, or $-(CH_2)_n-CH_3$;

m is 0, 1, 2, 3, 4, 5, or 6;

R^2 is a hydrophobic group selected from the group consisting of *n*-propyl, C_4-C_8 alkyl, C_2-C_8alkenyl, C_2-C_8alkynyl, C_3-C_8cycloalkyl, C_2-C_7heterocyclyl, aryl, heteroaryl, aralkyl, heteroaralkyl, and side chains of naturally occurring hydrophobic amino acids;

n is 0, 1, or 2; and

q is 0, 1, 2, 3, or 4.

In certain embodiments, q is 0, 1, or 2.

In certain other embodiments, q is 0.

In yet other embodiments, n is 0.

In still yet other embodiments, n is 1.

In certain other embodiments, n is 2.

In certain embodiments, X is $B(Y^1)(Y^2)$.

In certain other embodiments, X is $B(OH)_2$.

In certain embodiments, n is 1; q is 0; and X is $B(OH)_2$.

In certain embodiments, R^2 is selected from the group consisting of *t*-butyl, isobutyl, pentyl, cyclohexyl, benzyl, or naphthyl.

In certain other embodiments, R^2 is selected from the group consisting of *t*-butyl, isobutyl, or pentyl.

In still yet other embodiments, R^2 is *t*-butyl.

In certain embodiments, R^2 is the side chain of a naturally occurring hydrophobic amino acid.

In certain other embodiments, R^2 is the side chain of leucine, isoleucine, *tert*-leucine, phenylalanine, or tryptophan.

In certain embodiments, the compound of Formula I is *t*-butylGly-boroPro.

In certain embodiments, the stereochemical configuration at the carbon bearing X is L.

In certain other embodiments, the stereochemical configuration at the carbon bearing X is D.

In certain embodiments, the stereochemical configuration at the carbon bearing R^2 is L.

In certain other embodiments, the stereochemical configuration at the carbon bearing R^2 is D.

In certain embodiments, the stereochemical configuration at the carbon bearing X is L; and the stereochemical configuration at the carbon bearing R^2 is L.

In certain other embodiments, the stereochemical configuration at the carbon bearing X is L; and the stereochemical configuration at the carbon bearing R^2 is D.

In yet other embodiments, the stereochemical configuration at the carbon bearing X is D; and the stereochemical configuration at the carbon bearing R^2 is L.

In certain embodiments, the stereochemical configuration at the carbon bearing X is D; and the stereochemical configuration at the carbon bearing R^2 is D.

The term "DASH serine protease" means dipeptidyl peptidase (DPP) IV activity and/or structural homologues thereof. These proteins are enzymes that are united by their common post-proline-cleaving serine dipeptidase mechanism. For example, DPP-VII, originally named quiescent cell proline dipeptidase (QPP), is a DASH serine protease.

Val-boroPro, also known as PT-100 or talabostat, appears to stimulate immunity via the activation of caspase-1 and induction of IL-1β in macrophages, which in turn upregulates cytokine and chemokine expression in macrophages and stromal fibroblasts. Intracellular DPP 8 and/or 9 activity appears to be the relevant target for PT-100 in macrophages. This mechanism of action indicates a hitherto unforeseen regulatory role for intracellular DPPs in the immune system.

ARI-4175, tertiary-butyl (abbreviated *t*-butyl) Gly-boroPro, is a dipeptide boronic acid that potently inhibits all six members of the prolyl peptidase family of serine proteases as an adjuvant for dendritic cell vaccines for the treatment of cancer. Similarly to PT-100, ARI-4175 inhibits DPP8/9 activity. Other dipeptide boronic acids, preferably with a bulky, hydrophobic side chain such as isoleucine-boroPro, butylglycine-boroPro, phenylalanine-boroPro (Phe-boroPro), and cyclohexylglycine-boroPro (Cyg-boroPro) are expected to perform in a similar way. Routine experimentation by one skilled in the art could determine which compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) could be used successfully in the claimed methods.

PT-100 activates tumor immunity in mice via cytokine/chemokine upregulation in tumors and draining lymph nodes. The use of cytokines as cancer vaccine adjuvants is not new: e.g., GM-CSF for sipuleucel-T and GM-CSF or IL-2 and IFN-γ for vaccines in development. However, in comparison to these applications, the orally active DPP8/9 inhibitors, PT-100 and ARI-4175, have the advantage of stimulating tumor-associated macrophages and stromal cells to produce a combination of cytokines and chemokines that can cooperate to activate tumor-specific effector T cells. Among the cytokines and chemokines upregulated by PT-100, IL-1β, CXCL9 and CXCL10 are particularly noteworthy. IL-1β produced by tumor-associated macrophages plays a pivotal role in activating proinflammatory responses and in promoting development of T_h17 cells in the tumor microenvironment.

Based on strong preclinical antitumor activity and a novel mechanism of action, PT-100 was advanced into human trials in cancer and granted fast-track designation by the FDA. However, despite some signals of clinical activity in non-randomized Phase II studies of non-Hodgkin's lymphoma (NHL), metastatic melanoma and non-small cell lung cancer (NSCLC), PT-100 ultimately failed to meet its goals in pivotal Phase III trials in NSCLC. Two factors are likely to have contributed to this failure. Most importantly, preclinical studies indicated that for optimal antitumor activity of PT-100 in mice, an endogenous immune response to the tumor is required. It was unlikely that any such underlying tumor immunity remained in the late-stage NSCLC patients studied in Phase III. Secondly, dose-limiting toxicity in cancer patients appeared to prevent administration of high enough PT-100 doses for consistent immune stimulation patient to patient. The studies by Fry *et al.* suggest that PT-100's mechanism of action should be most effective clinically when employed to boost cancer vaccines. It is possible that PT-100 might be clinically successful when used with an appropriate vaccine that can prime tumor-specific T cells; but it is the goal of the present invention to identify an analog with lower toxicity that will achieve clinical success in humans.

ARI-4175 or other compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I), alone or in combination with dendritic cell therapy (DCT), trastuzamab, cetuximab, ipilimumab, vemurafenib, sorafenib, or other cancer immunotherapies, have a significant advantage over other cancer immunotherapies because they are orally active small molecules. They elicit immune activation similar to difficult and expensive DCT or antibody treatments, but can be administered much more easily. ARI-4175 and compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) are the first "orally active tumor antigens" of their kind.

Many patients do not respond to cetuximab, or develop resistance after initial response to therapy. This is due to the cancer developing resistance to the immune system of the patient. The immune response is still present, but is either no longer strong enough to kill the tumor or the tumor becomes invisible to the immune system. One example is the KRAS mutation found in approximately 40% of malignant colorectal cancers. A recent clinical trial (Lièvre *et al.*, *Cancer Res.* **2006**, 66 (8), 3992) found that KRAS mutations were correlated with resistance to cetuximab, while all of the patients who responded to cetuximab lacked the KRAS mutation. Although the molecular mechanisms by which

cetuximab produces a clinical response remain unknown, re-activation of the immune response with ARI-4175 may, alone or in combination with cetuximab or other immunotherapies, improve clinical outcomes in patients whose immune response is insufficient to kill the tumor or who have a refractory cancer.

ARI-4175 or other compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) may also be used in combination with T cell adoptive transfer therapies. This treatment method uses T-cell-based cytotoxic responses to attack cancer cells. The adjuvant properties of ARI-4175 would allow it to be used as a pre-treatment before administration of tumor-infiltrating lymphocytes, or TIL, or as a post-treatment after administration of adoptive cell transfer.

The low toxicity of ARI-4175 (or other compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) allows it to be used as an adjuvant in cancer patients whose cancer is currently in remission. Such patients would benefit from an increased anti-tumor immune response to avoid relapse.

It is expected that ARI-4175 and other compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) would perform synergistically with CTLA4 inhibitors, such as ipilimumab (Yervoy®), which is a receptor antagonist that increases immune activity. The CTLA4 (cytotoxic T lymphocyte antigen 4), also known as CD152, is a protein receptor that downregulates the immune system. ARI-4175 or other compounds that inhibit a plurality of mammalian DASH serine proteases (e.g., compounds of Formula I) in combination with CD28 receptor agonists would work in a similar way to increase the activity of T cells.

Exemplification

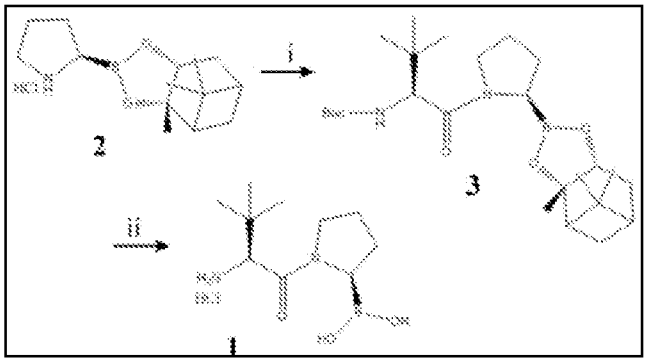
The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1

Rationale, Synthesis of Compounds, and Inhibition of RMS Tumor Growth

Synthesis of ARI-4175. Commercially available L-boroPro-pn **2** was coupled to an *N*-Boc protected unnatural amino acid Boc-Tle-OH **3** (CAS NO 62965-35-9) using HATU to render a protected dipeptide boronate Boc-Tle-boroPro-pn. Concurrent removal of both

protection groups by trichloroborane (BCl_3) followed by reverse-phase HPLC purification yields the desired product **1** (ARI-4175) as an HCl salt.



Scheme 1. Synthetic scheme for ARI-4175. Reagents and conditions: (i) Boc-L-Tle-OH, HATU/DIPEA/DMF, (ii) BCl_3.

Synthesis of PT-100. PT-100 was synthesized as previously described in sufficient quantities for the studies described in the following examples.

ARI-4175 is a nanomolar inhibitor of the DPP-IV-like serine proteases, including DPPs 8 and 9 (Table 1), which are the putative targets in PT-100's immune mechanism of action. Inhibition of DPP-IV and FAP activity may also contribute to the antitumor effect of PT-100 because selective abrogation of DPP-IV or FAP activity appears to slow tumor growth.

Table 1. ***Potency of inhibition of DPP-IV-like protease activities in vitro***

Inhibitor	IC_{50}[1] (nM)					
	DPP-IV	DPP8	DPP9	DPP-II	FAP	PREP[2]
PT-100 (Val-boroPro)	0.7	3.6	1.7	8.2	17.0	35.0
ARI-4175 (t-butylGly-boroPro)	1.6	5.1	1.9	88.0	32.0	24.0

[1]50% mean inhibitory concentration

[2]Prolyl endopeptidase

C57BL/6 mice bearing established RMS were vaccinated intramuscularly in a hind limb with RMS DC vaccine on day 10 after tumor inoculation. ARI-4175, PT-100 or vehicle was administered by daily gavage from day 10 onwards for 3 cycles of 5 days each as described herein: ARI-4175, 10 mg/kg, cycle 1 and 5 mg/kg cycles 2 and 3; PT-100, 1 mg/kg cycles 1 to 3. Another group of mice received vaccine but no compounds and groups of unvaccinated mice received compounds or vehicle. 8 replicate mice were treated in each regimen.

As shown in Figure 8, administration of ARI-4175 by itself significantly slowed tumor growth (Fig. 8A) and produced tumor regression in 3/8 mice by day 25 (Fig. 9). In

combination with vaccine, ARI-4175 produced regression in 6/8 mice (Fig. 9) and increased mouse survival significantly (P = 0.0045; Fig 1B). In contrast, PT-100 administered at a dose of 1 mg/kg, either with or without DC vaccination, failed to produce tumor regression (Fig. 2) by day 25 or significant inhibition of tumor growth (Fig. 8A), and tumor growth was only reduced in 2 out of 6 mice treated with PT-100 and the vaccine (Fig. 9). The 1 mg/kg dose of PT-100 was previously shown to be optimal for activation of tumor immunity in C57BL/6 mice, and the dose cannot be increased much higher because the MTD for PT-100 is ~2 mg/kg in C57BL/6 mice. Therefore, the significant vaccine adjuvant effect of a 10/5-mg/kg dose of ARI-4175 suggests that ARI-4175 is less toxic than PT-100, and that it is possible to increase the dose of ARI-4175 to achieve greater tumor regression and mouse survival than is possible with PT-100 at tolerated doses in the RMS mouse model.

Example 2

Effectiveness of ARI-4175 in the RMS DC tumor vaccine model

Figure 17 A shows the experimental setup for priming of T cell donors and T cell recipients. Figure 17 B shows the tumor volume curve (mean ± standard deviation) and survival curve. Mice receiving ARI-4175 alone had significantly smaller tumors compared to saline (n=10, p=0.0019). Though ARI-4175 + primed T cell recipients had smaller tumors, the difference was not significant when compared to ARI-4175 + naïve T cell recipients (n=10, p=0.0755). Eight of ten ARI-4175 + primed T cell recipients survived to day 80, however this was not significant compared to the 40% survival of ARI-4175 + naïve T cell recipients (n=10, p=0.0658).

Figures 18 A and B show that combination treatment with ARI-4175 and adoptive T cell transfer in Rag1-/- recipients significantly reduces RMS M3-9-M volume. (A) Female $Rag1^{-/-}$ mice received naïve or RMS-primed T cells one day after tumor challenge. (B) By day 10, ARI-4175 treated mice had significantly smaller tumors than saline treated mice (naïve: n=5, p=0.0159; primed: n=5, p=0.0079). $Rag1^{-/-}$ mice treated with tumor-primed T cells in combination with ARI-4175 had the smallest tumors overall.

ARI-4175 is a potent adjuvant to DC vaccination as evidenced by significantly improved survival and reduced RMS M3-9-M volume (Figure 19). Figure 19 A shows a late treatment model for DC vaccination and ARI-4175 treatment. Figure 19 B shows the tumor volume curve (mean ± s.d.) and survival curve. Both groups treated with ARI-4175 had significantly improved survival compared to controls (sham vaccine: n=7, p<0.001, DC

vaccine: n=7, p<0.001). Mice treated with a combination therapy using ARI-4175 and DC vaccine had significantly smaller tumors compared to mice treated with ARI-4175 alone (n=7, p=0.0481).

The RMS cell line was derived from *Ink4a/Arf*$^{-/-}$ mice transgenic for hepatocyte growth factor/scatter factor (HGF/SF) that develop malignant RMS with high penetrance. DC vaccine will be prepared by incubating bone marrow derived DCs with apoptotic bodies generated from RMS cells as previously described. Tumor growth was monitored by caliper measurement every 2 days.

Example 3

Induction of IL-1β and upregulation of cytokine and chemokine expression in tumors and draining lymph nodes by Ala-boroPro (2243), Val-boroPro (PT-100, 2054), and *t*-BuGly-boroPro (ARI-4175)

Method for Cytokine Assay in BALB/c Mice. Female BALB/c mice were treated with various doses of PT-100 or ARI-4175 by oral gavage (PO) or intraperitoneal (IP) injection and serum was analyzed for chemokines (Figures 4, 5, and 6). Blood was collected by cardiac puncture at various times post-dose and serum was prepared for analysis by ELISA. The samples were assayed for mouse mouse cytokines G-CSF and mouse CXCL1 using ELISA kits from R&D Systems (Cat. No. MCS00 and MKC00B respectively). All measurements were made in duplicate. Serum samples were diluted as necessary to obtain values within the range of the assays. Optimal dilutions required varied depending on the test agent and can range from no dilution for control samples or test agents with no activity to 1:1000 dilution for very high samples. For agents that produce a positive response the strongest signal was observed at 2 hrs post-dose for CXCL1 and at 6 hours post-dose for G-CSF. The dose response varies with test agent but 20 μg/mouse dose is an acceptable baseline dose for response assessment. Typically 6 animals are measured for each agent at each time point.

Figure 4 demonstrates that Val-boroPro (2054), but not Ala-boroPro (2243), stimulates G-CSF and CXCL1/KC in BALB/c mice. G-CSF and CXL1/KC are markers of anti-cancer immune enhancing activity.

ARI-4175 (4175-2 in Figures 5 and 6) is a very potent inducer of cytokines. Stimulation of G-CSF increased until 6 h after administration; CXCL1 was rapidly induced at 3 h after administration but had disappeared by 6 h (Figure 5). ARI-4175 (4175-2) is at

least 5 times more potent than PT-100 (2054) at inducing G-CSF and CXCL1 cytokines (Figure 6). Serum from ARI-4175 treated mice also had increased IL-18, IL-1β and IFN-γ compared to vehicle but at much lower levels than G-CSF and CXCL1.

PT-100 stimulates expression of proinflammatory cytokine and chemokine mRNA 2 hours after oral administration to tumor-bearing mice. The PT-100 response is characterized by upregulation of IL-1β, G-CSF, IL-6, CXCL1, CXCL9 and CXCL10 in tumor and lymph node tissue. ARI-4175 was recently found to stimulate development of IL-17 producing T_h17 cells *in vitro* (V. Kuchroo, unpublished data). T_h-17 cells appear to contribute to effective antitumor immunity in certain cancers; therefore, IL-17 will be included with the cytokine/chemokine panel characterizing the response to PT-100 that we will investigate after administration of ARI-4175 and PT-100 to RMS-DC vaccinated mice. RNA expression will be assayed in RMS tumor and draining lymph node tissues 2 hours after compound administration at optimal doses. We will use the RT-PCR procedure used previously to analyze the upregulation of cytokines and chemokines by PT-100 in mice bearing A549 lung carcinoma xenografts. cDNA will be synthesized with an iScript kit (Biorad, Hercules, CA) from total RNA extracted by Trizol (Invitrogen, Carlsbad, CA), diluted 1:10 in water, and amplified for 40 cycles in a thermal cycler (cDNA denaturing, 95 °C/15 s; annealing and extension, 60 °C/30 s) using 10-μM unlabeled primer pairs in a 2X iQ Sybergreen Supermix (Biorad). Reactions will use Taqman probes 5′-labeled with HEX, FAM or Texas Red and 3′-labeled with black hole quenchers (Biosearch Technologies, Novato, CA). Cytokine/chemokine target mRNA and 18s RNA reference control forward/reverse primer pairs and Taqman probes will be designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). mRNA copy numbers will be calculated from cycle threshold values using standard curves with size-characterized reference cDNA, which will be synthesized and amplified from mouse tissue RNA, purified by electrophoresis and a gel purification kit (Qiagen, Valencia, CA), and quantified by PicoGreen (Invitrogen, Carlsbad, CA).

As shown in Figures 11 and 12, treatment of mice with ARI-4175 also increased NK cell expression of the FcγRIII receptor, CD16 and the degranulation marker LAMP-1. *In vitro* treatment of human NK cells also increased the activation marker, CD69 (Figure 15). The therapeutic effect of ARI-4175 might partially be due to the augmentation of ADCC through elevating expression of CD16 (FcγRIIIA) and activating NK cells (based on CD69 upregulation).

Example 4

Immunological memory by tumor rechallenge of vaccinated mice in which tumor regression and rejection occurs

An effective vaccine for cancer would have the advantage of establishing immunological memory that can protect against disseminated metastasis or tumor regrowth following clinical response to initial treatment. Mice in which intramuscular RMS tumors are rejected after RMS DC vaccination followed by ARI-4175 or PT-100 treatment in Example 1 were rechallenged by intramuscular injection of 10 x 10^6 RMS cells at least 20-30 days after rejection of primary tumors. Mice were monitored for secondary tumor growth for a further 20-30 days without any additional therapeutic treatment. In order to demonstrate immunological specificity of protection, mice will also be challenged with the C57BL/6 tumor cell line, EL4 (ATCC, TIB-39). Groups of 4 mice were tested for immunological memory. Similar experiments demonstrating tumor-specific memory following PT-100 treatment have been described.

As shown in Figure 7, female C57BL/6 mice were challenged intramuscularly with 1x10^6 RMS M3-9-M on day 0. Mice were orally gavaged with 200 μg ARI-4175 on days 3-7, 17-21, 24-28, and 31-35. Tumor-free survivors were rechallenged on day 56 with 5x10^5 RMS M3-9-M and monitored with no additional 4175 treatment. Following rechallenge, RMS M3-9-M showed initial growth followed by rejection in all mice (n=7, p=0.0175).

Example 5

Assay tumor-specific CTL in RMS DC vaccinated mice

CTL will be assayed *ex vivo* by the ^{51}Cr-release assay in tumor-draining lymph nodes and spleens of RMS tumor-bearing mice receiving the RMS DC vaccine and ARI-4175 or PT-100 treatments described in Example 1. The assay will be performed as previously described for the measurement of tumor-specific CTL responses stimulated by PT-100 in EL4 tumor-inoculated C57BL/6 mice. Specificity of CTLs will be investigated by comparing cytotoxicity against RMS versus EL4 cells.

Example 6

MTDs of ARI-4175 and PT-100 in C57BL/6 mice

Previously, in C57BL/6 mice administered PT-100 at doses above the MTD (~2 mg/kg/day), no obvious signs of toxicity were observed prior to death; therefore, the end point for determination of MTD was mortality in this experiment. In order to gain insight into the causes of toxicity, histopathology was investigated to determine the dose response

of plasma cytokine and chemokine levels. The cytokine/chemokine assay was used to determine whether toxicity is related to PT-100's putative mechanism of action. Preliminary experiments indicated that blockade of cytokine/chemokine responses with the IL-1R antagonist, anakinra, does not alter the toxicity of PT-100 in rats, suggesting that the toxicity of PT-100 may not be due to systemic cytokine/chemokine production.

Summary of Rat Toxicity. A maximum tolerated dose (MTD) study was undertaken to compare the MTD for ARI-4175 (*t*-BuGly-boroPro) and PT-100 (Val-boroPro, ARI-2054) in Sprague Dawley rats. Animals were dosed by either subcutaneous injection (SQ) or oral gavage (PO). Six animals were used at each dose. A blood sample was drawn from the tail at 30 minutes post-dose which was used to measure plasma drug concentrations. The starting dose for both compounds was 0.05 mg/kg body weight and then the dose was adjusted up or down for each drug to determine the maximum dose at which there was 100% survival. At the starting dose of 0.05 mg/kg body weight SQ, both ARI-4175 and PT-100 treatment resulted in at least one animal death within the first 24 hours. A 0.01 mg/kg body weight dose, SQ, did not result in any deaths and no adverse effects were observed for 48 hours post-dose. A third experiment was done at a dose of 0.025 mg/kg body weight which resulted in one death in the ARI-4175 group but no deaths in the PT-100 group. The study was repeated with oral dosing starting again at 0.05 mg/kg body weight. Both drugs were apparently more tolerated by the oral dose as there were no adverse effects in this experiment at 0.05 mg/kg PO. Increasing the dose to 0.1 mg/kg PO resulted in 2 deaths in the PT-100 group but no adverse effects with ARI-4175. At 0.25 mg/kg ARI-4175 PO there was one death. Therefore the MTD observed for SQ was 0.025 mg/kg for PT-100 and 0.01 mg/kg for ARI-4175. By the oral route the MTD was 0.05 mg/kg for PT-100 and 0.1 mg/kg for ARI-4175. Evaluation of the plasma drug concentrations suggests toxicity results at equivalent plasma drug concentrations regardless of the route of administration. Toxicity is observed for the dose of drug that results in plasma drug concentration of 100 +/-50 nM. This data is summarized in Figure 20.

Mouse Toxicity Plasma vs. Dose Figures. C57BL/6 mice were treated by oral gavage at various doses up to 40 mg/kg of ARI-4175 and up to 10 mg/kg of PT-100 (ARI-2054) daily for 5 days. On the 5th day blood was drawn pre-dose and at 30 minutes post-dose and plasma drug concentrations were measured by LCMS. Oral availability of PT-100 is 3-4 times greater than that of ARI-4175 as evidenced by the plasma concentrations at the 10 mg/kg dose. Plasma concentrations are approximately proportional to dose over the

range tested. Survival was 100% in this experiment but all groups showed significant weight loss over the 5 day treatment period. The results are shown in Figure 21.

PK for 4175 in Mice. Pharmacokinetics of ARI-4175 was measured in normal BALB/c mice in both the open (linear) and closed (cyclic) forms of the drug by oral gavage (PO) and by, intraperitoneal (IP), subcutaneous (SQ) and intravenous (IV) injection. The open form of the drug was prepared by incubation of the drug at room temperature overnight at pH 2. The closed form was prepared by incubation overnight at pH 7.4 (in PBS). The open (linear) samples were neutralized by dilution into PBS immediately before administration. The treatment groups are listed below (Table 2).

Table 2

Group	Dose	Route	n
1	1 mpk	PO	4
2	1 mpk	IP	4
3	1 mpk	SQ	4
4	0.5 mpk	IV	6

Blood was collected from the tail vein for all groups except the IV groups. The IV injection was made in the tail vein and therefore blood was collected from a distant site (submandibular vein). Plasma samples were prepared and the concentration of ARI-4175 in each sample was measured by LC-MS. The results are shown in Figure 22.

Example 7

Investigation of histopathology in mice receiving escalating doses of ARI-4175 and PT-100

Groups of 3 mice will be inoculated intramuscularly with RMS cells and administered PT-100 and ARI-4175 by gavage at doses increasing from the MED determined in Example 1 up to the MTD determined in Example 6. One 5-day cycle of each compound will be given from day 10 to day 14 after tumor inoculation, and on day 18, specimens of tumor, draining lymph node, spleen, liver, lung and kidney tissue will be fixed in formalin and embedded in paraffin. H&E stained tissue sections from test mice will be compared histologically with sections from control mice. PT-100 has been shown to stimulate leukocytic infiltration of solid tumors. The tumor infiltrates are characterized by neutrophils concentrated at the borders of tumor and stromal tissue. Comparison of tumor sections from ARI-4175 treated mice versus sections from PT-100 treated mice will determine whether ARI-4175 also promotes tumor infiltration. It is possible that the

toxicity of PT-100 results from leukocytic infiltration of non-tumor tissues leading to inflammatory responses that cause organ failure. Therefore, we will examine the non-tumor tissue samples for the presence of leukocytes in mice treated with PT-100 and ARI-4175.

Example 8

Investigation of role of systemic cytokines/chemokines in toxicity using IL-1 receptor deficient mice

Cytokine/chemokine responses to PT-100 are abrogated in IL-1R1 deficient B6.129S7-*Il1r1*^{tm1Imx}/J mice (Jackson Laboratory); therefore, if toxicity is due to activity of systemic cytokines/chemokines, MTD should be significantly increased in IL-R1 mutant mice relative to congenic C57BL/6 mice. Therefore, the dose responses of serum G-CSF and CXCL1 cytokines will be compared by ELISA (R&D Systems) and the MTDs of ARI-4175 and PT-100 in B6.129S7-*Il1r1*^{tm1Imx}/J versus C57BL/6 control mice. MTDs will be determined in groups of 3 mice treated at increasing dose levels. G-CSF and CXCL-1 levels will be determined in serum sampled at 3 and 8 hours after compound administration. If IL-1R1 deficient mice are resistance to toxicity, and if histopathology in Experiment 6 reveals leukocytic infiltration of non-tumor tissue, IL-1R1 deficient and sufficient mice will be compared histologically to determine if toxicity is related to inflammatory disruption of organ function.

Example 9

Potential anti-tumor and immunologic effects of ARI-4175 in KRAS mutated colorectal cancer cell lines; co-administration of ARI-4175 with cetuximab

Cetuximab (CTX) is an effective therapeutic agent in a number of malignancies. Current data indicate that about 40% of colorectal cancer patients bearing mutated K-ras do not benefit from this agent. A possible mechanism of the antitumor effect of cetuximab is mediated through antibody-dependent cell-mediated cytotoxicity (ADCC). This study investigated the potential of activity of ARI-4175, in the treatment of K-ras mutant colorectal cancer xenografts as a single agent or in combination with cetuximab.

The effect of ARI-4175 alone or in combination with cetuximab was evaluated both *in vitro* and *in vivo*. *In vitro*, the proliferation of K-ras mutant colon cancer cell lines DLD-1 and HCT-116 was detected after three days of culture in the medium containing various concentrations of ARI-4175 or cetuximab (Figure 10). ARI-4175 (10 nM−200 μM) alone or in combination with cetuximab did not show significant cytotoxicity on either DLD-1 or

HCT-116 in cell culture (Figure 10). *In vivo*, nude mice bearing DLD-1 or HCT-116 xenograft tumors were randomly divided into four groups, control, ARI-4175 alone, cetuximab alone and ARI-4175 plus cetuximab. ARI-4175 was administered orally at 100 μg, q.d or b.i.d and cetuximab was injected intraperitoneally at 200 μg per week. Tumor measurements were conducted twice a week. In mice growth of both DLD-1 and HCT-116 tumors were significantly blocked by the application of ARI-4175 in a dose-dependent manner (Figure 13 A and B). The combination of ARI-4175 with cetuximab led to a further decrease in tumor size although not statistically significant, probably due to lower number of animals. Cetuximab alone did not show any therapeutic effect on HCT-116 xenograft but did have moderate efficacy on DLD-1 tumors.

Example 10

Comparison of pharmacokinetic profile of PT-100 and ARI-4175; other differences between the two compounds

As shown in Figure 16, female C57BL/6 mice were challenged subcutaneously with $1x10^6$ MB49. Mice were orally gavaged on days 3-7 and 10-14. Tumor volume was monitored by caliper measurements. At a 20 μg dose, both PT-100 and ARI-4175 induce anti-tumor activity. ARI-4175 dosing at 200 μg induced full regression in 5 of 5 mice whereas PT-100 was toxic at the same dose.

Despite the small differences in chemical structure between PT-100 and ARI-4175, there are unexpectedly large differences in the pharmacokinetic (PK) profile of the two compounds. In particular, the toxicity of ARI-4175 is much lower.

Incorporation By Reference

All of the U.S. patents and U.S. patent application publications cited herein are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We Claim:

1. A method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

2. The method of claim 1, wherein said compound is not Val-boroPro.

3. The method of claim 1 or 2, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

4. The method of claim 1, 2, or 3, wherein the cancer is selected from the group consisting of basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain cancer, breast cancer, cervical cancer, choriocarcinoma, CNS cancer, colon and rectum cancer, connective tissue cancer, cancer of the digestive system, endometrial cancer, esophageal cancer, eye cancer, cancer of the head and neck, gastric cancer, intra-epithelial neoplasm, kidney cancer, larynx cancer, leukemia, acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, chronic lymphoid leukemia, liver cancer, small cell lung cancer, non-small cell lung cancer, lymphoma, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, melanoma, myeloma, neuroblastoma, oral cavity cancer, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, rectal cancer, renal cancer, cancer of the respiratory system, sarcoma, skin cancer, stomach cancer, testicular cancer, thyroid cancer, uterine cancer, and cancer of the urinary system.

5. The method of claim 1, 2, or 3, wherein the cancer is selected from the group consisting of prostate cancer, colorectal cancer, multiple myeloma, and non-small cell lung cancer.

6. The method of claim 1, 2, or 3, wherein the cancer is selected from lung cancer, colorectal cancer, breast cancer, pancreatic cancer and prostate cancer.

7. The method of claim 1, 2, or 3, wherein the cancer is lung cancer.

8. The method of claim 7, wherein the cancer is non-small cell lung cancer.

9. The method of claim 1, 2, or 3, wherein the cancer is colorectal cancer.

10. The method of claim 1, 2, or 3, wherein the cancer is breast cancer.

11. The method of claim 1, 2, or 3, wherein the cancer is pancreatic cancer.

12. The method of claim 1, 2, or 3, wherein the cancer is prostate cancer.

13. The method of any one of the preceding claims, wherein the cancer is metastatic.

14. The method of any one of the preceding claims, further comprising co-administering to the mammal a therapeutically effective amount of tumor-primed T-cells.

15. The method of claim 14, wherein said tumor-primed T-cells are administered prior to said administration of the compound.

16. The method of claim 14, wherein said tumor-primed T-cells are administered subsequent to said administration of the compound.

17. The method of claim 14, wherein said tumor-primed T-cells are administered concurrently to said administration of the compound.

18. The method of any one of claims 1-13, further comprising co-administering to the mammal a therapeutically effective amount of an orally active tumor antigen.

19. The method of any one of claims 1-13, further comprising co-administering to the mammal a therapeutically effective amount of a dendritic cell vaccine.

20. The method of any one of the preceding claims, further comprising administration of an adjuvant.

21. The method of any one of claims 1-13, further comprising treating the mammal with a second therapy selected from the group consisting of surgery, radiation and chemotherapy.

22. The method of claim 21, wherein the second therapy is surgery.

23. The method of claim 21, wherein the second therapy is radiation.

24. The method of claim 21, wherein the second therapy is chemotherapy.

25. The method of claim 24, wherein the chemotherapy is selected from the group consisting of ipilimumab, vemurafenib, GDC-0879, PLX-4720, aldesleukin, asparaginase, bleomycin sulfate, carboplatin, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin hydrochloride, docetaxel, doxorubicin, doxorubicin hydrochloride, epirubicin hydrochloride, etoposide, etoposide phosphate, floxuridine, fludarabine, fluorouracil, gemcitabine, gemcitabine hydrochloride, hydroxyurea, idarubicin hydrochloride, ifosfamide, interferons, interferon-α2a, interferon-α2b, interferon-αn3, interferon-α1b, interleukins, irinotecan, mechlorethamine hydrochloride, melphalan, mercatopurine, methotrexate, methotrexate sodium, mitomycin, mitoxantrone, paclitaxel, pegaspargase, pentostatin, prednisone, profimer sodium, procabazine hydrochloride, taxol, taxotere, teniposide, topotecan hydrochloride, vinblastine sulfate, vincristine sulfate and vinorelbine tartrate.

26. The method of claim 24, wherein the chemotherapy is selected from the group consisting of bleomycin sulfate, carboplatin, cisplatin, docetaxel, doxorubicin, doxorubicin

hydrochloride, fluorouracil, gemcitabine, gemcitabine hydrochloride, methotrexate, methotrexate sodium, paclitaxel, taxol, taxotere, vinblastine sulfate and vincristine sulfate.

27. The method of claim 24, wherein the chemotherapy is a dipeptidylpeptidase IV inhibitor.

28. The method of claim 24, wherein the chemotherapy is a FAP-activated chemotherapeutic, a FAP-activated dipeptidylpeptidase IV inhibitor, or a FAP-activated proteasome inhibitor.

29. The method of claim 24, wherein the chemotherapy is a FAP-activated proteasome inhibitor.

30. The method of claim 24, wherein the chemotherapy is an antibody.

31. The method of claim 30, wherein the antibody is selected from the group consisting of trastuzamab, cetuximab, bevacizumab, and rituximab.

32. A method of increasing antitumor immunity in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

33. The method of claim 32, wherein said compound is not Val-boroPro.

34. The method of claim 32 or 33, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

35. The method of claim 32, 33, or 34, wherein said antitumor immunity is increased for tumors selected from the group consisting of lung tumors, lymphomas, breast tumors, colorectal tumors, thyroid tumors, uterine tumors, pancreatic tumors, prostate tumors, skin tumors, kidney tumors, liver tumors and brain tumors.

36. The method of claim 32, 33, or 34, wherein said antitumor immunity is increased for tumors selected from the group consisting of lung tumors, breast tumors, colorectal tumors, pancreatic tumors and prostate tumors.

37. The method of any one of claims 32-36, wherein the antitumor immunity comprises antibody-dependent cell-mediated cytotoxicity.

38. A method of stimulating or enhancing an immune response in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

39. The method of claim 38, wherein said compound is not Val-boroPro.

40. The method of claim 38 or 39, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

41. The method of claim 38, 39, or 40, wherein said immune response is stimulated.

42. The method of claim 38, 39, or 40, wherein said immune response is enhanced.

43. The method of any one of claims 38-42, wherein the immune response comprises antibody-dependent cell-mediated cytotoxicity.

44. The method of any one of claims 38-43, wherein the mammal has cancer or is at risk of developing cancer.

45. The method of any one of claims 38-43, wherein said mammal is in remission of cancer.

46. The method of any one of claims 38-43, wherein said mammal has a refractory or resistant cancer.

47. A method of treating a condition characterized by abnormal cell proliferation, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

48. The method of claim 47, wherein said compound is not Val-boroPro.

49. The method of claim 47 or 48, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

50. The method of claim 47, 48, or 49, wherein the abnormal cell proliferation is cancer, a blood vessel proliferative disorder or a fibrotic disorder.

51. The method of claim 47, 48, or 49, wherein the abnormal cell proliferation is abnormal angiogenesis.

52. A method of increasing cytokine and/or chemokine production in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases.

53. The method of claim 52, wherein said compound is not Val-boroPro.

54. The method of claim 52 or 53, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

55. A method of stimulating or enhancing production of T-cells in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases, wherein said T-cells recognize an antigen on a malignant cell.

56. The method of claim 55, wherein said compound is not Val-boroPro.

57. The method of claim 55 or 56, wherein said compound induces the production of a cytokine selected from the group consisting of GCSF and CXCL1.

58. The method of claim 55, 56, or 57, wherein said production of T-cells is stimulated.

59. The method of claim 55, 56, or 57, wherein said production of T-cells is enhanced.

60. The method of any one of claims 55-59, wherein said malignant cell is a carcinoma, sarcoma, leukemia, lymphoma or myeloma.

61. The method of any one of the preceding claims, wherein the mammal is a primate, canine, equine, feline or bovine.

62. The method of any one of the preceding claims, wherein the mammal is a human.

63. The method of any one of the preceding claims, wherein the compound is administered orally or parenterally.

64. The method of claim 63, wherein the compound is administered parenterally.

65. The method of claim 63, wherein the compound is administered orally.

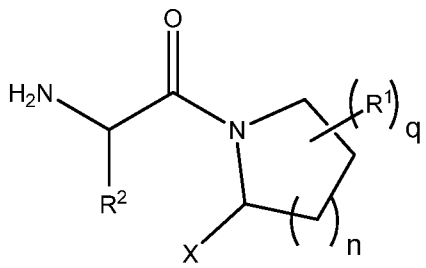
66. The method of claim 65, wherein the compound is administered in a solid dosage form.

67. The method of claim 66, wherein the solid dosage form is a tablet, capsule or pill.

68. The method of claim 66, wherein the solid dosage form is a tablet.

69. The method of any one of the preceding claims, wherein the compound is administered in an amount sufficient to stimulate the immune system without dose limiting toxicity.

70. The method of any one of claims 1-69, wherein the compound is represented by Formula I:



Formula I

wherein:

X is $B(Y^1)(Y^2)$ or CN;

Y^1 and Y^2 are independently OH, or together with the boron atom to which they are attached represent a group that is hydrolysable to a boronic acid, or together with the boron atom to which they are attached form a 5- to 8-membered ring that is hydrolysable to a boronic acid;

R^1 is selected from the group consisting of halogen, lower alkyl, lower alkenyl, lower alkynyl, carbonyl, carboxyl, ester, formate, ketone, thiocarbonyl, thioester, thioacetate, thioformate, amino, acylamino, amido, nitro, sulfate, sulfonate, sulfonamido, $-(CH_2)_m-R_7$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_7$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, or $-(CH_2)_n-S-(CH_2)_m-R_7$, azido, cyano, isocyanato, thiocyanato, isothiocyanato, cyanato,  , or  ;

R_7 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle;

R_8 independently represents hydrogen, $-CH_3$, or $-(CH_2)_n-CH_3$;

m is 0, 1, 2, 3, 4, 5, or 6;

R^2 is a hydrophobic group selected from the group consisting of *n*-propyl, C_4-C_8 alkyl, C_2-C_8alkenyl, C_2-C_8alkynyl, C_3-C_8cycloalkyl, C_2-C_7heterocyclyl, aryl, heteroaryl, aralkyl, heteroaralkyl, and side chains of naturally occurring hydrophobic amino acids;

n is 0, 1, or 2; and

q is 0, 1, 2, 3, or 4.

71. The method of claim 70, wherein q is 0, 1, or 2.

72. The method of claim 70, wherein q is 0.

73. The method of any one of claims 70-72, wherein n is 0.

74. The method of any one of claims 70-72, wherein n is 1.

75. The method of any one of claims 70-72, wherein n is 2.

76. The method of any one of claims 70-75, wherein X is $B(Y^1)(Y^2)$.

77. The method of any one of claims 70-75, wherein X is $B(OH)_2$.

78. The method of claim 70, wherein n is 1; q is 0; and X is $B(OH)_2$.

79. The method of any one of claims 70-78, wherein R^2 is selected from the group consisting of *t*-butyl, isobutyl, pentyl, cyclohexyl, benzyl, or naphthyl.

80. The method of any one of claims 70-78, wherein R^2 is selected from the group consisting of *t*-butyl, isobutyl, or pentyl.

81. The method of any one of claims 70-78, wherein R^2 is *t*-butyl.

82. The method of any one of claims 70-78, wherein R^2 is the side chain of a naturally occurring hydrophobic amino acid.

83. The method of any one of claims 70-78, wherein R^2 is the side chain of leucine, isoleucine, tert-leucine, phenylalanine, or tryptophan.

84. The method of claim 70, wherein the compound of Formula I is *t*-butylGly-boroPro.

85. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is L.

86. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is D.

87. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing R^2 is L.

88. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing R^2 is D.

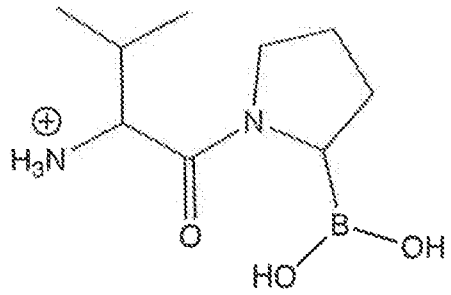
89. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is L; and the stereochemical configuration at the carbon bearing R^2 is L.

90. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is L; and the stereochemical configuration at the carbon bearing R^2 is D.

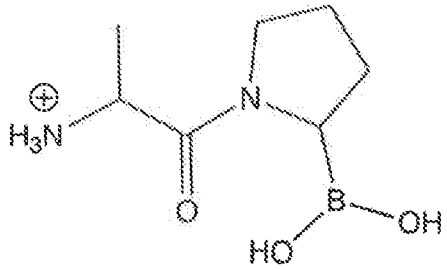
91. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is D; and the stereochemical configuration at the carbon bearing R^2 is L.

92. The method of any one of claims 70-84, wherein the stereochemical configuration at the carbon bearing X is D; and the stereochemical configuration at the carbon bearing R^2 is D.

Figure 1



Val-boroPro

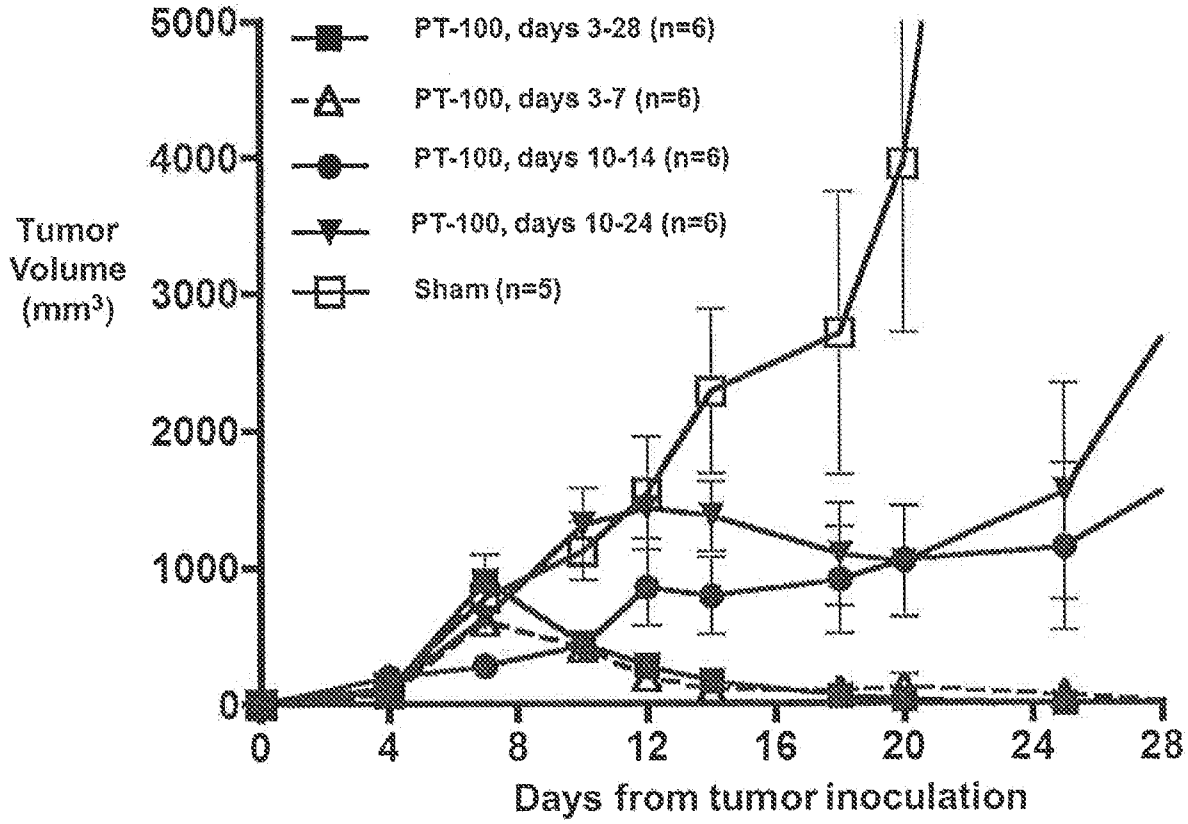


Ala-boroPro

	K_i / IC50* (nM)							
	DPPIV	**DPP8**	**DPP9**	**DPPII**	**FAP**	**PREP**	**MTD (mg/kg)**	**Immune Stimulation**
V-bP	0.18	1.5	0.76	8.2*	17*	35*	0.05	G-CSF, IL-1β, INFγ
A-bP	0.027	2.0	0.53	1.4*	43*	240*	>200	No

In Vitro IC_{50} (nM)					
Compound	**DPPIV**	**DPP8**	**DPP9**	**DPPII**	**FAP**
PT-100	0.7	3.6	1.7	8.2	17
ARI-4175	1.6	5.1	1.9	88	32

Figure 2


5000
4000
3000
2000
1000
0
Tumor Volume (mm³)
PT-100, days 3-28 (n=6)
PT-100, days 3-7 (n=6)
PT-100, days 10-14 (n=6)
PT-100, days 10-24 (n=6)
Sham (n=5)
0
4
8
12
16
20
24
28
Days from tumor inoculation

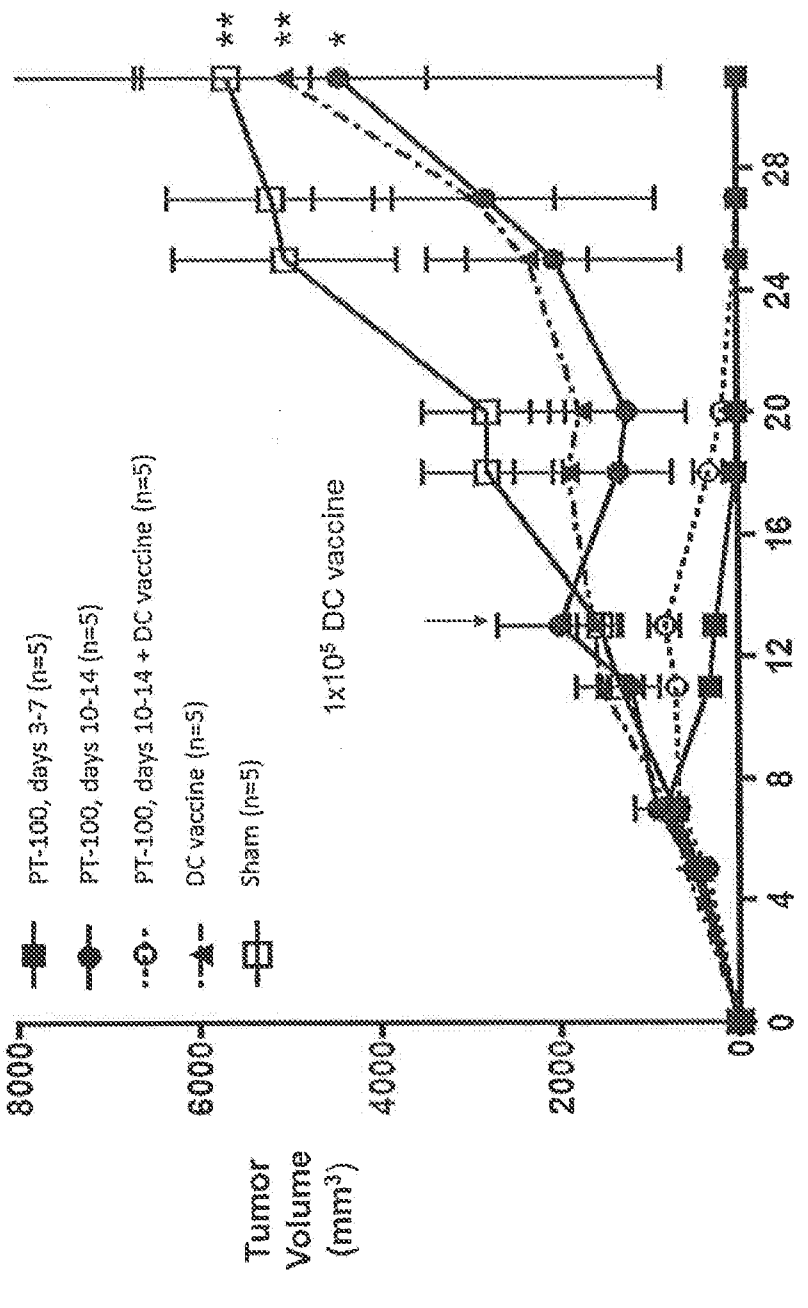

Tumor Volume (mm³)
8000
6000
4000
2000
0
0
4
8
12
16
20
24
28
1x10⁵ DC vaccine
PT-100, days 3-7 (n=5)
PT-100, days 10-14 (n=5)
PT-100, days 10-14 + DC vaccine (n=5)
DC vaccine (n=5)
Sham (n=5)
*
**
**

Figure 3

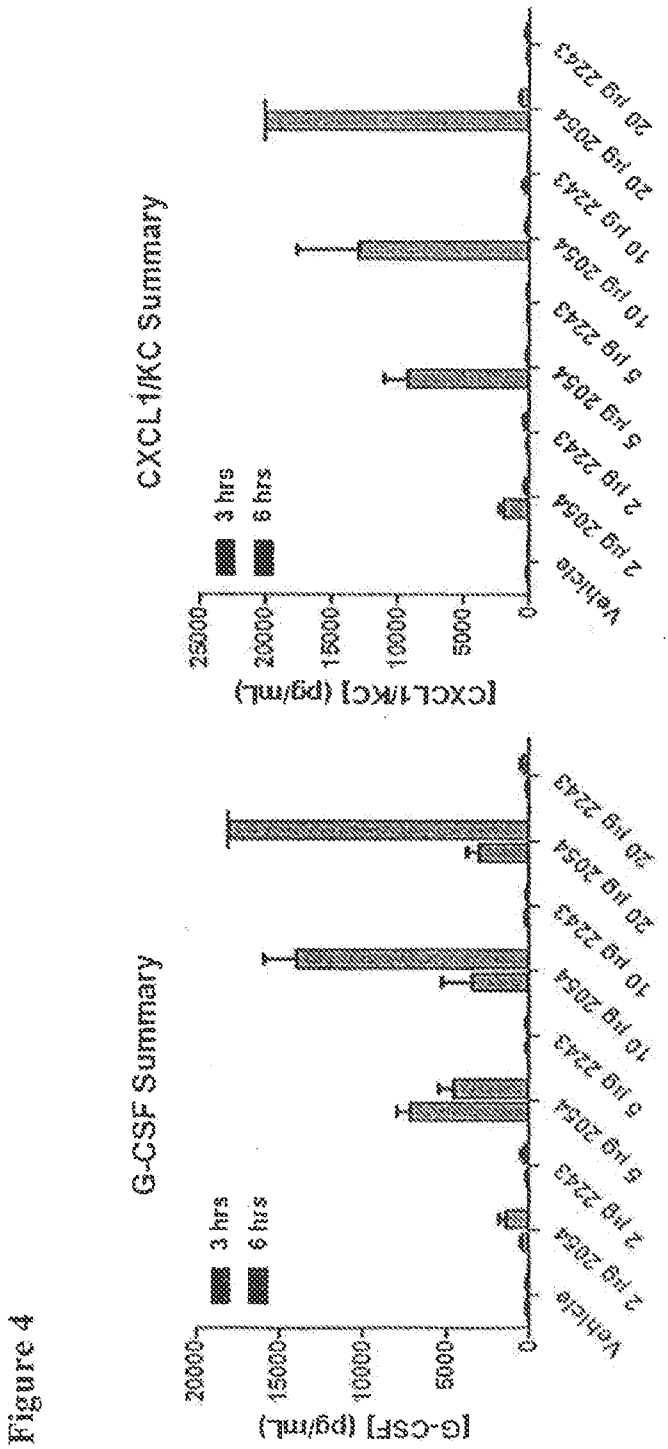

G-CSF Summary
3 hrs
6 hrs
[G-CSF] (pg/mL)
20000
15000
10000
5000
0
Vehicle
2 µg 20Sa
2 µg 22A3
5 µg 20Sa
5 µg 22A3
10 µg 20Sa
10 µg 22A3
20 µg 20Sa
20 µg 22A3
CXCL1/KC Summary
3 hrs
6 hrs
[CXCL1/KC] (pg/mL)
25000
20000
15000
10000
5000
0
Vehicle
2 µg 20Sa
2 µg 22A3
5 µg 20Sa
5 µg 22A3
10 µg 20Sa
10 µg 22A3
20 µg 20Sa
20 µg 22A3

Figure 4

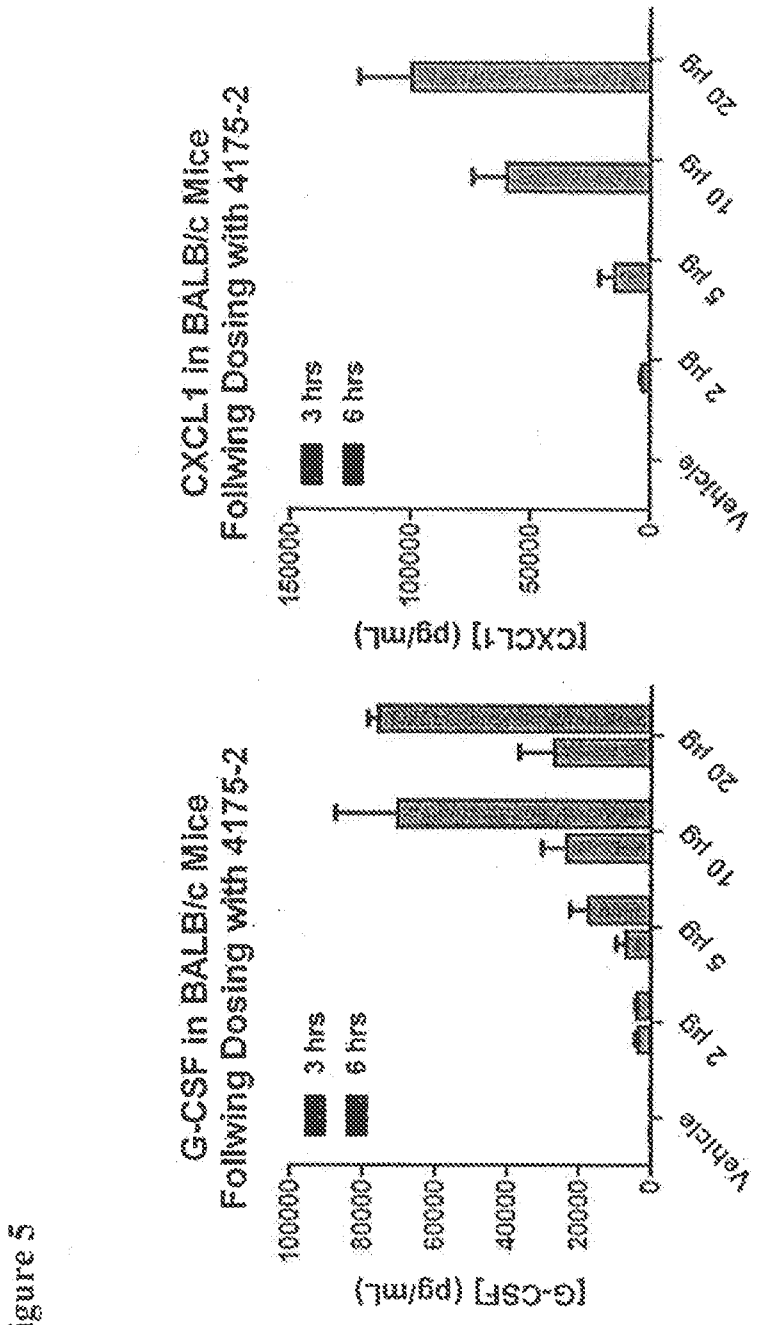

CXCL1 in BALB/c Mice
Follwing Dosing with 4175-2
3 hrs
6 hrs
[CXCL1] (pg/mL)
150000
100000
50000
0
Vehicle
2 µg
5 µg
10 µg
20 µg
G-CSF in BALB/c Mice
Follwing Dosing with 4175-2
3 hrs
6 hrs
[G-CSF] (pg/mL)
100000
80000
60000
40000
20000
0
Vehicle
2 µg
5 µg
10 µg
20 µg

Figure 5

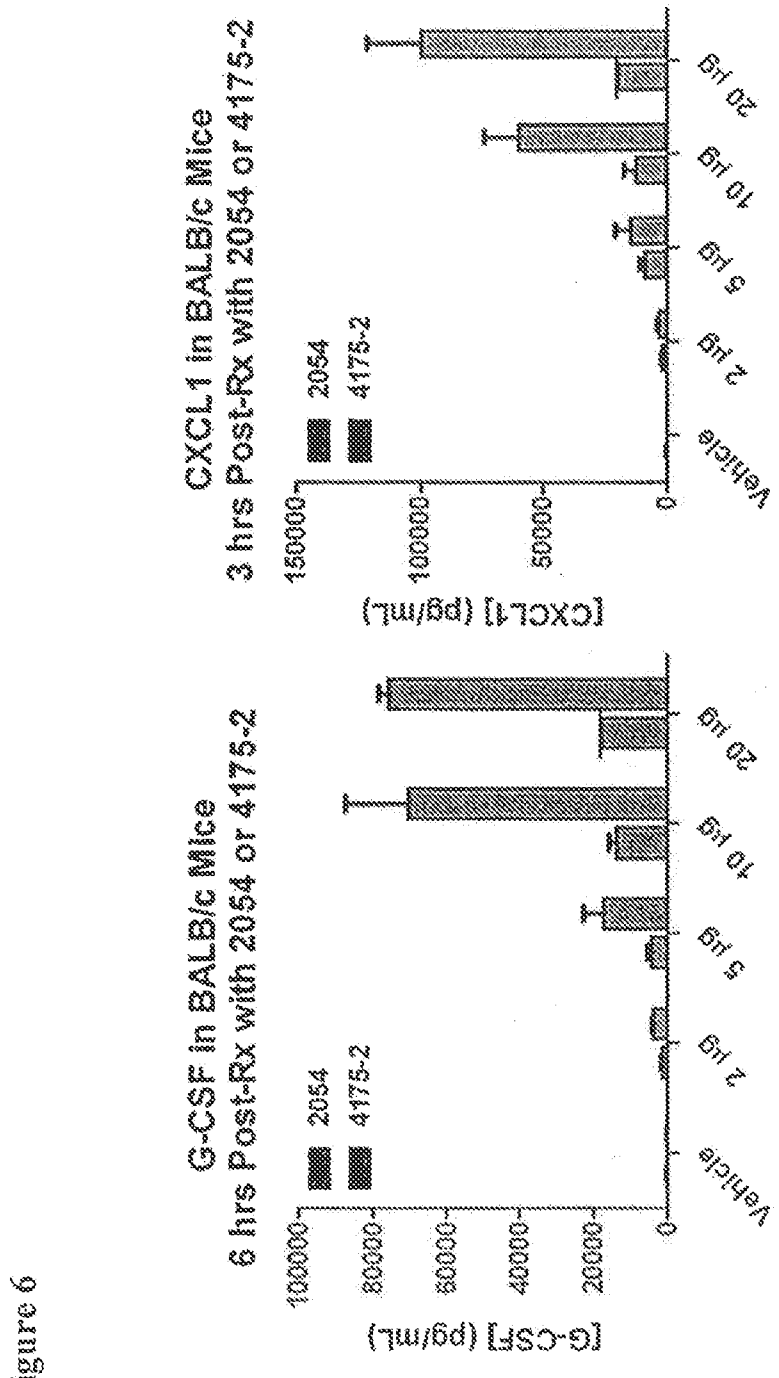

CXCL1 in BALB/c Mice
3 hrs Post-Rx with 2054 or 4175-2
[CXCL1] (pg/mL)
150000
100000
50000
0
2054
4175-2
Vehicle
2 mg
5 mg
10 mg
20 mg
G-CSF in BALB/c Mice
6 hrs Post-Rx with 2054 or 4175-2
[G-CSF] (pg/mL)
100000
80000
60000
40000
20000
0
2054
4175-2
Vehicle
2 mg
5 mg
10 mg
20 mg

Figure 6

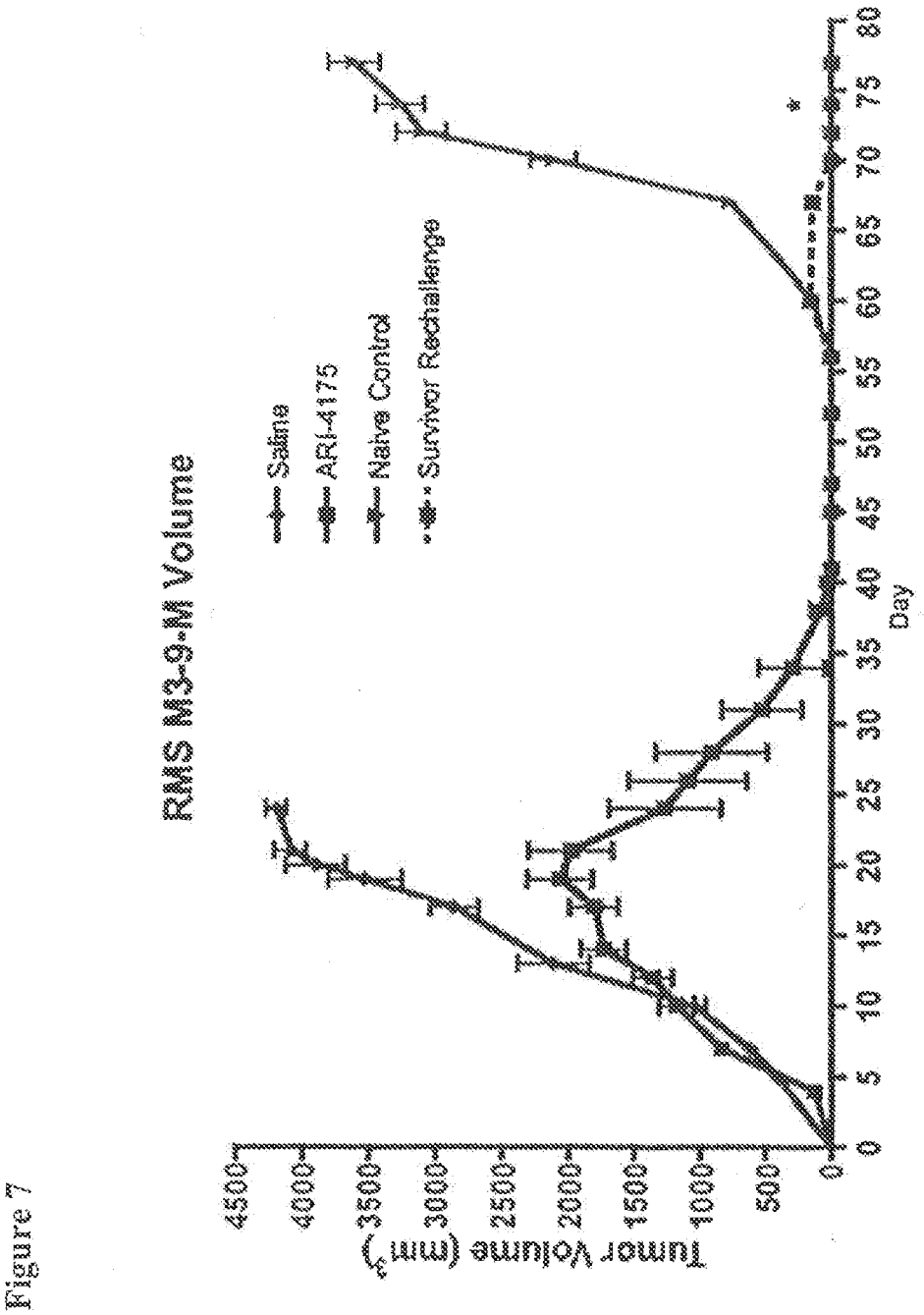

RMS M3-9-M Volume
Saline
ARI-4175
Naive Control
Survivor Rechallenge
Tumor Volume (mm³)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Day
*

Figure 7

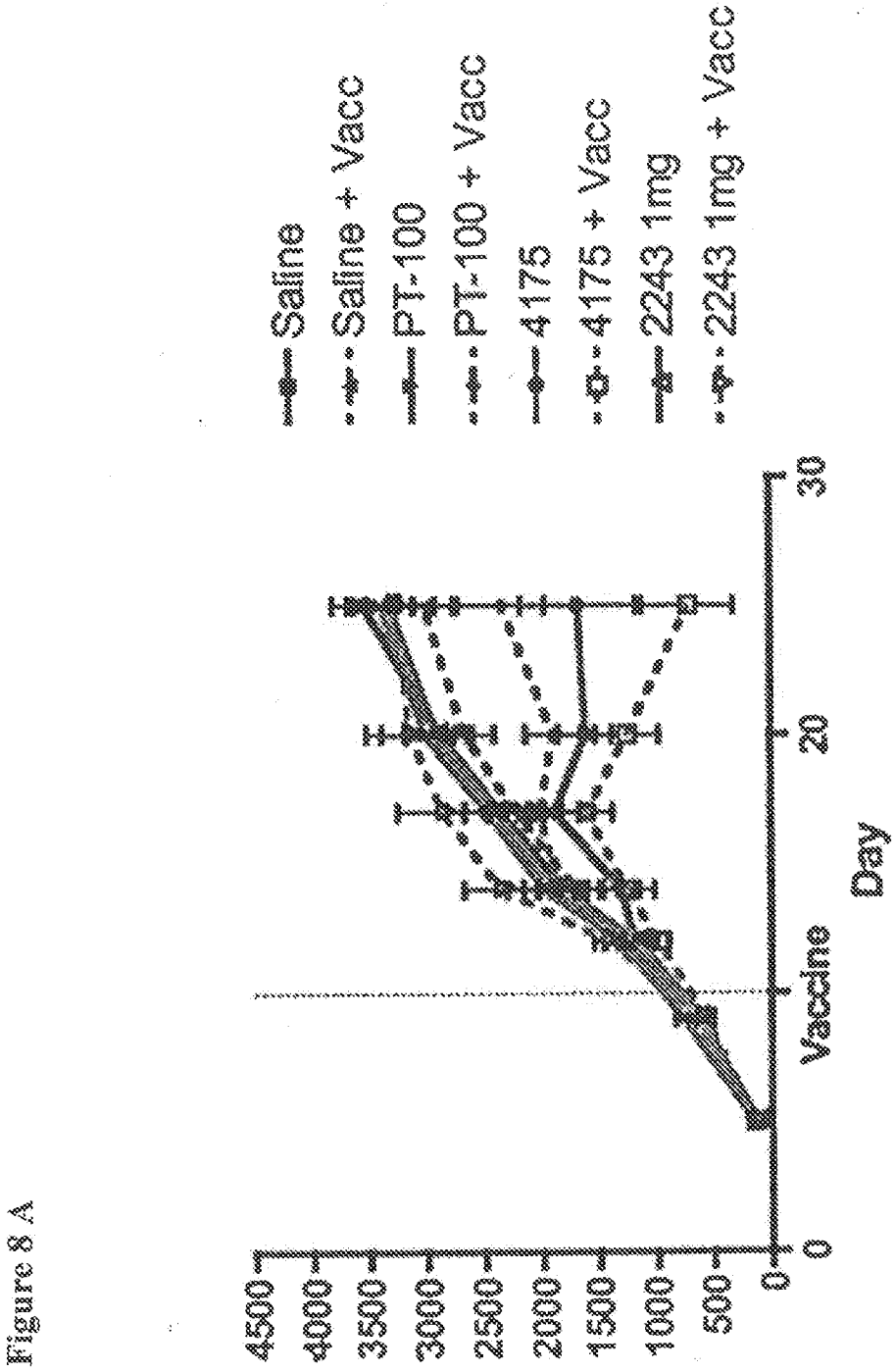

Saline
Saline + Vacc
PT-100
PT-100 + Vacc
4175
4175 + Vacc
2243 1mg
2243 1mg + Vacc
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
30
Vaccine
Day

Figure 8 A

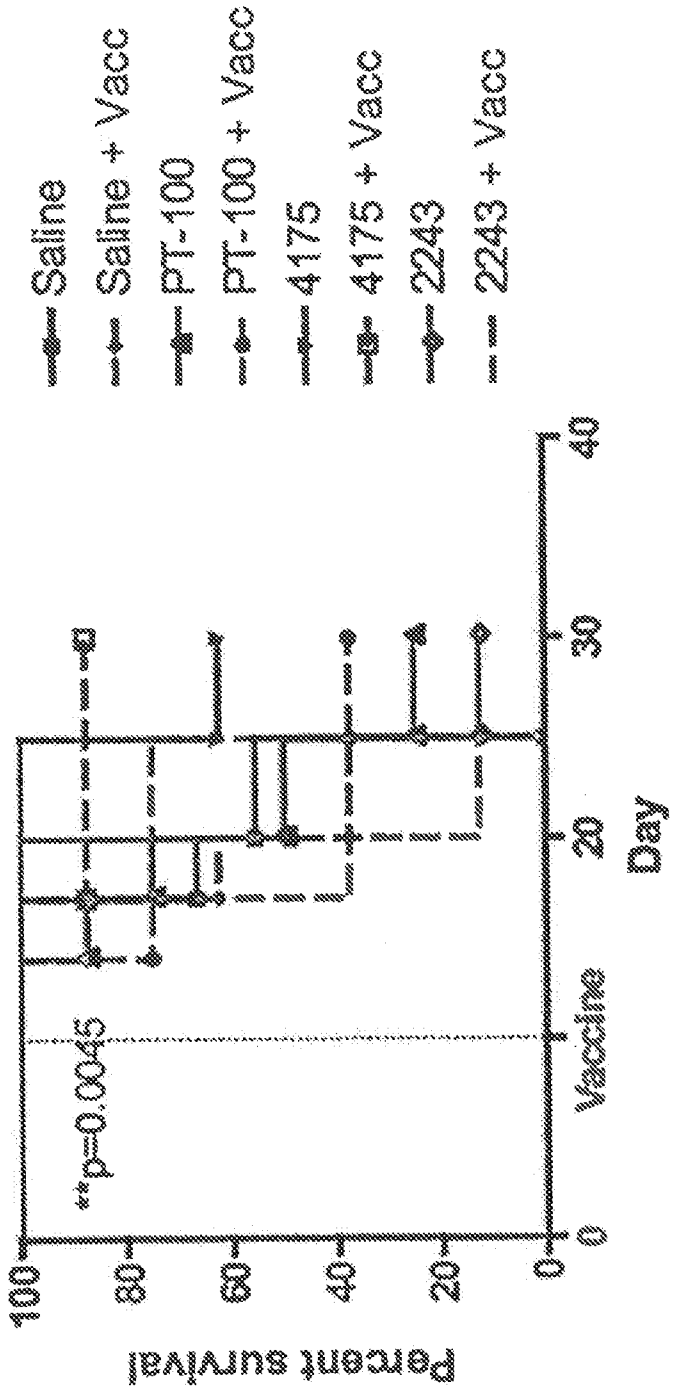

Saline
Saline + Vacc
PT-100
PT-100 + Vacc
4175
4175 + Vacc
2243
2243 + Vacc
Percent survival
100
80
60
40
20
0
**p=0.0045
Vaccine
0
20
30
40
Day

Figure 8 B

Figure 9

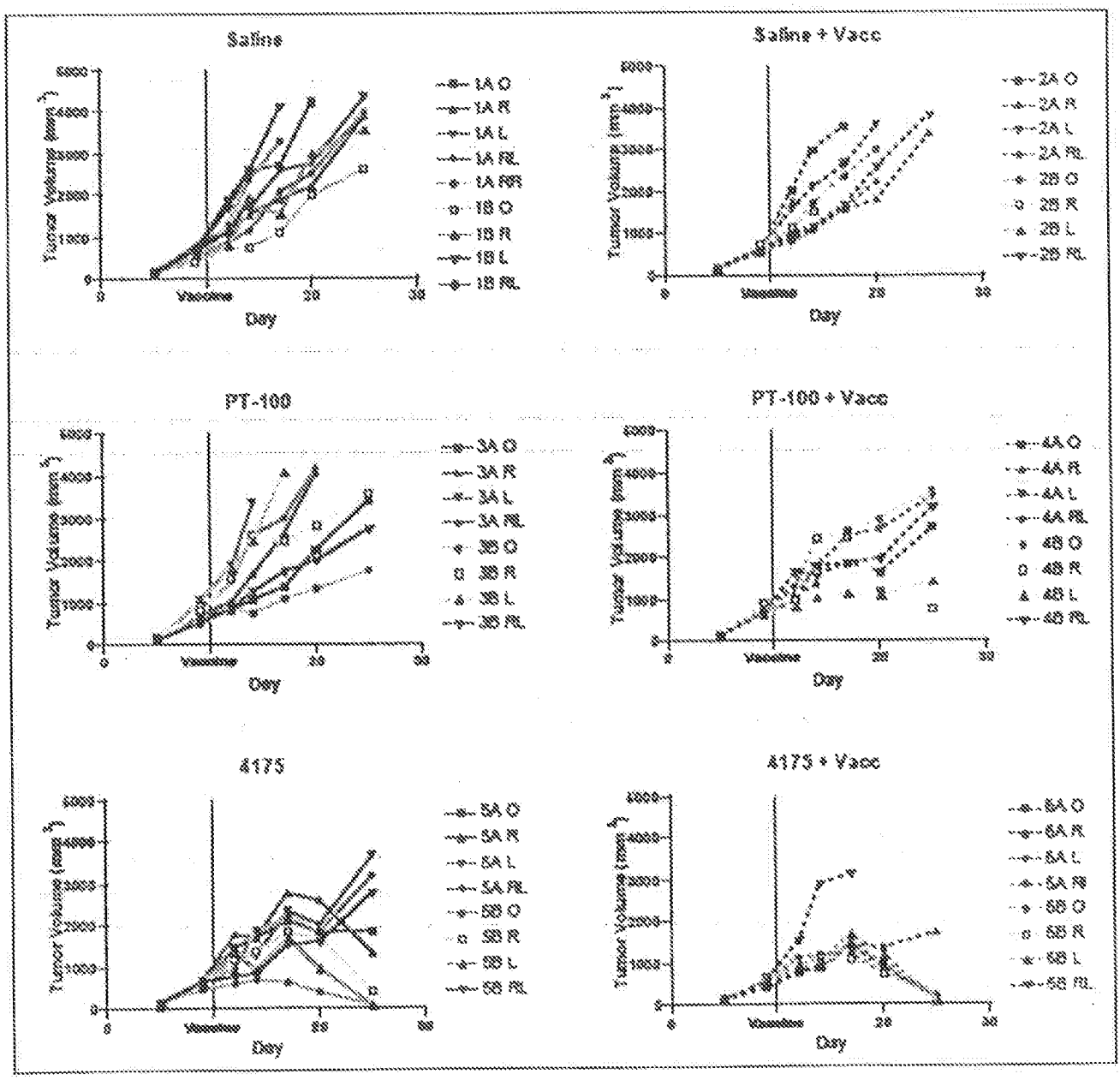

Saline
Saline + Vacc
PT-100
PT-100 + Vacc
4175
4175 + Vacc
Day
Vaccine

Figure 10

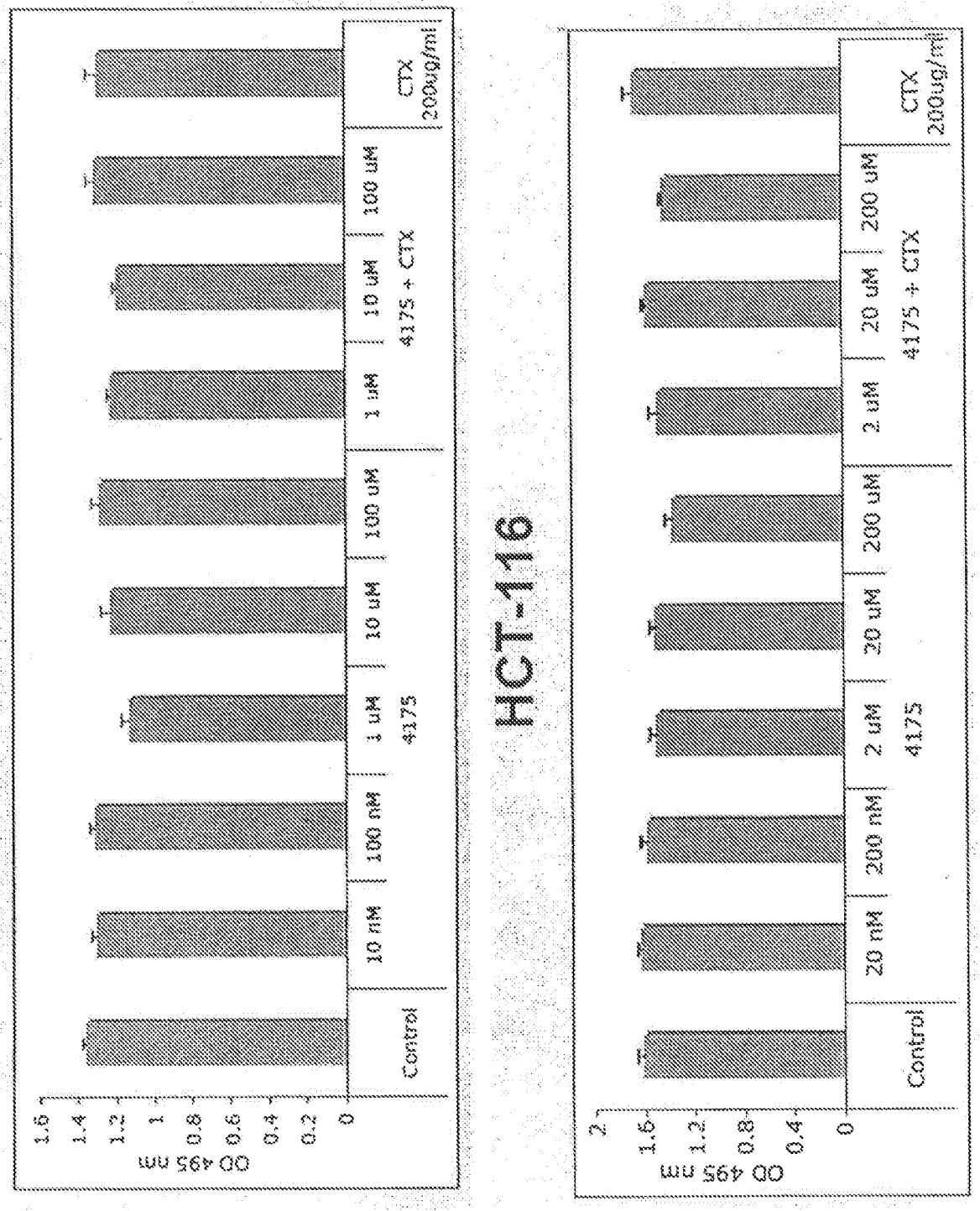

DLD1
OD 495 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
10 nM
100 nM
1 uM
10 uM
100 uM
4175
1 uM
10 uM
100 uM
4175 + CTX
CTX 200ug/ml
HCT-116
OD 495 nm
2
1.6
1.2
0.8
0.4
0
Control
20 nM
200 nM
2 uM
20 uM
200 uM
4175
2 uM
20 uM
200 uM
4175 + CTX
CTX 200ug/ml

Figure 11

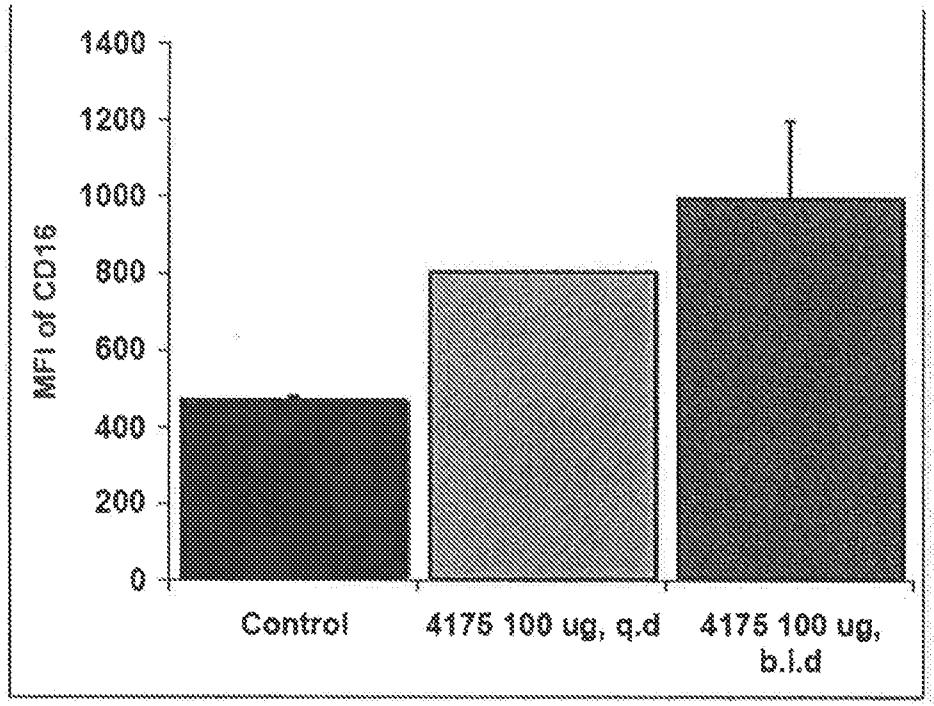


Figure 12

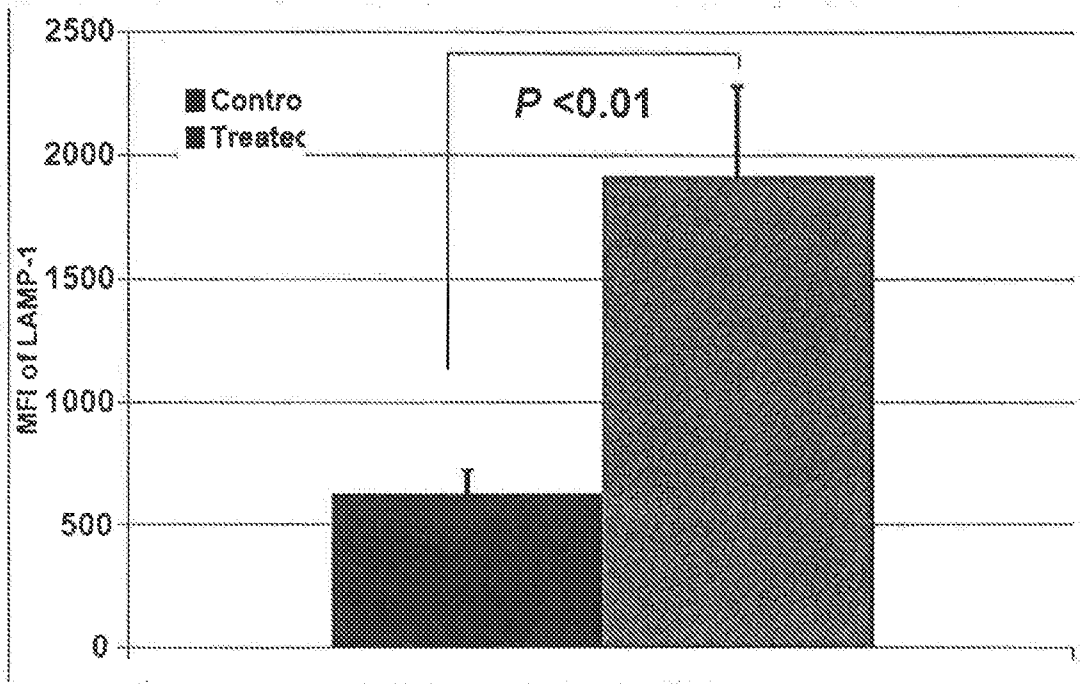
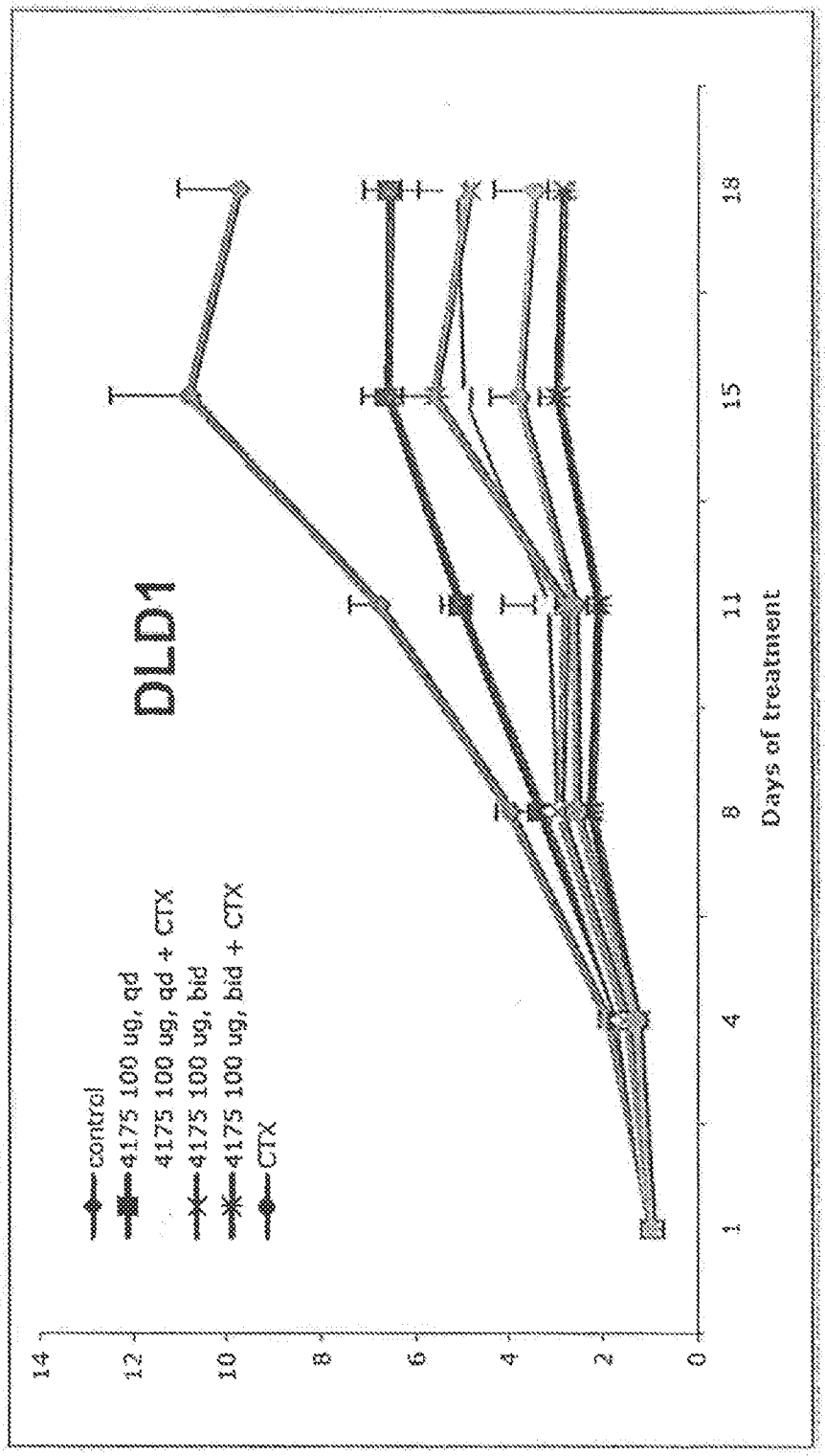


Figure 13 A


DLD1
control
4175 100 ug, qd
4175 100 ug, qd + CTX
4175 100 ug, bid
4175 100 ug, bid + CTX
CTX
Days of treatment
0
2
4
6
8
10
12
14
1
4
8
11
15
18

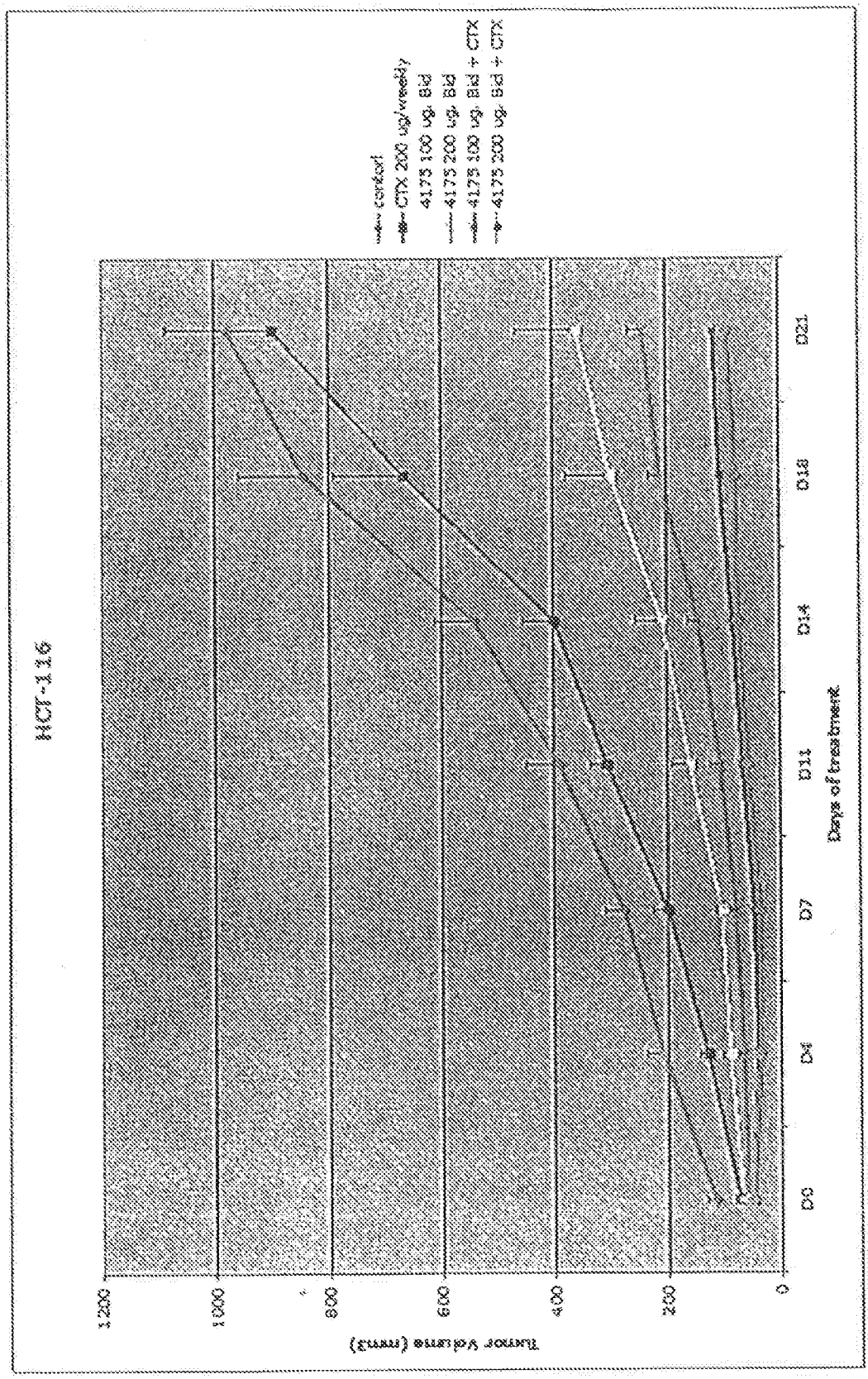
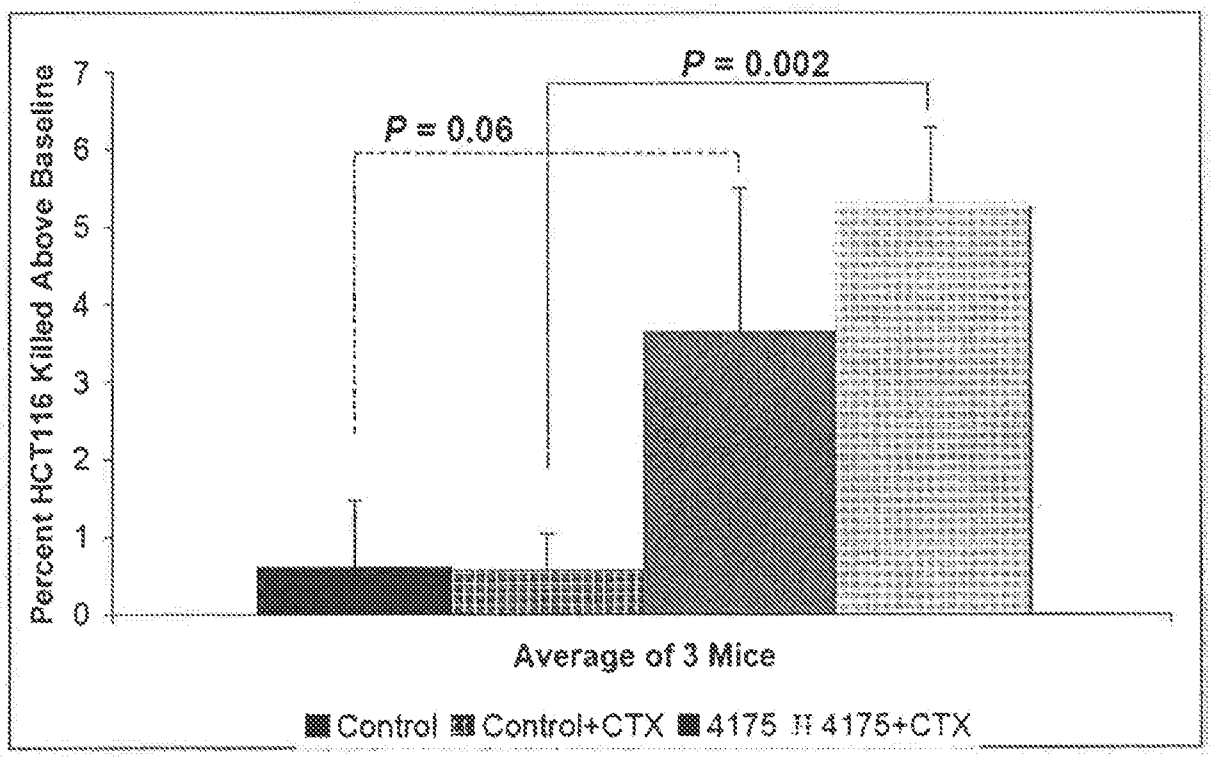

HCT-116
control
CTX 200 ug/weekly
4173 100 ug, Bid
4173 200 ug, Bid
4173 100 ug, Bid + CTX
4173 200 ug, Bid + CTX
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
D0
D4
D7
D11
D14
D18
D21
Days of treatment

Figure 13 B

Figure 14


P = 0.002
P = 0.06
Percent HCT116 Killed Above Baseline
0
1
2
3
4
5
6
7
Average of 3 Mice
Control
Control+CTX
4175
4175+CTX

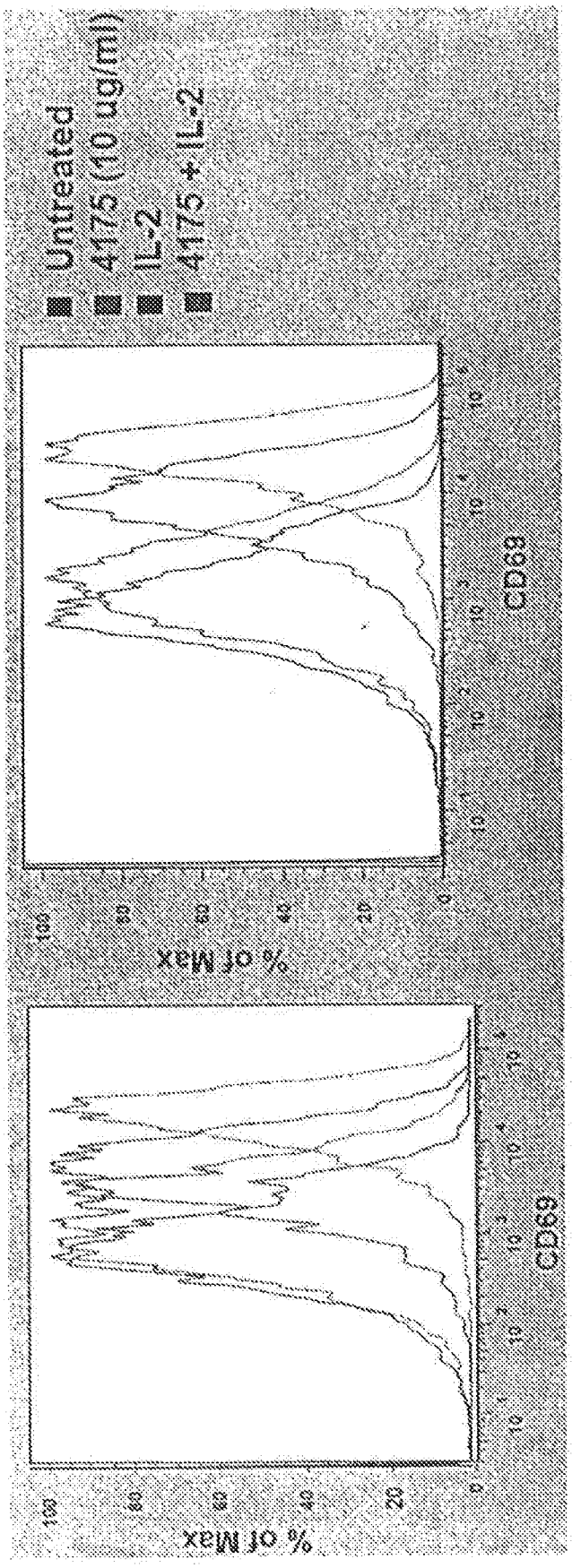
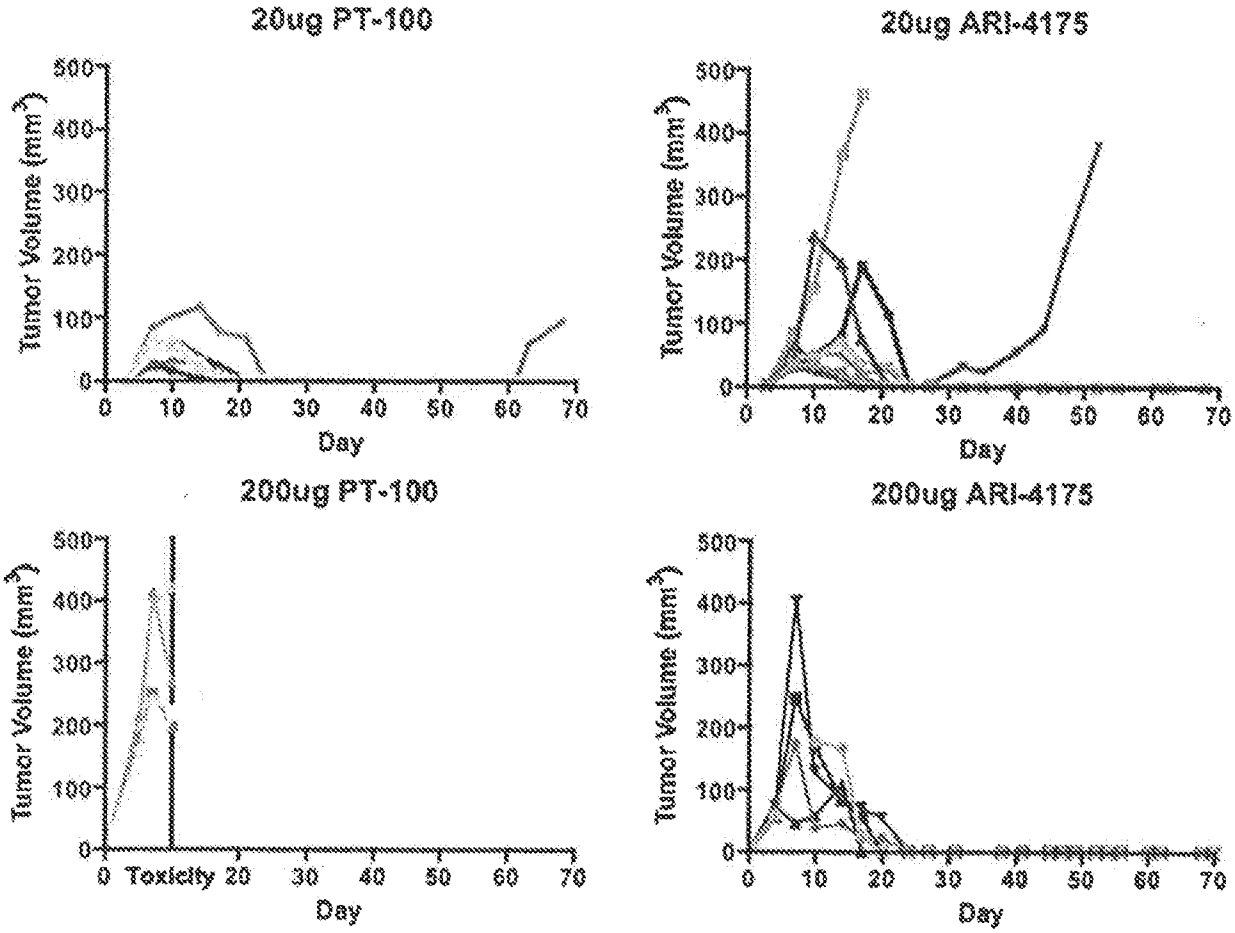


Figure 15

Figure 16


20ug PT-100
20ug ARI-4175
200ug PT-100
200ug ARI-4175
Tumor Volume (mm3)
Day
Toxicity
0
100
200
300
400
500
10
20
30
40
50
60
70

Figures 17 A and B

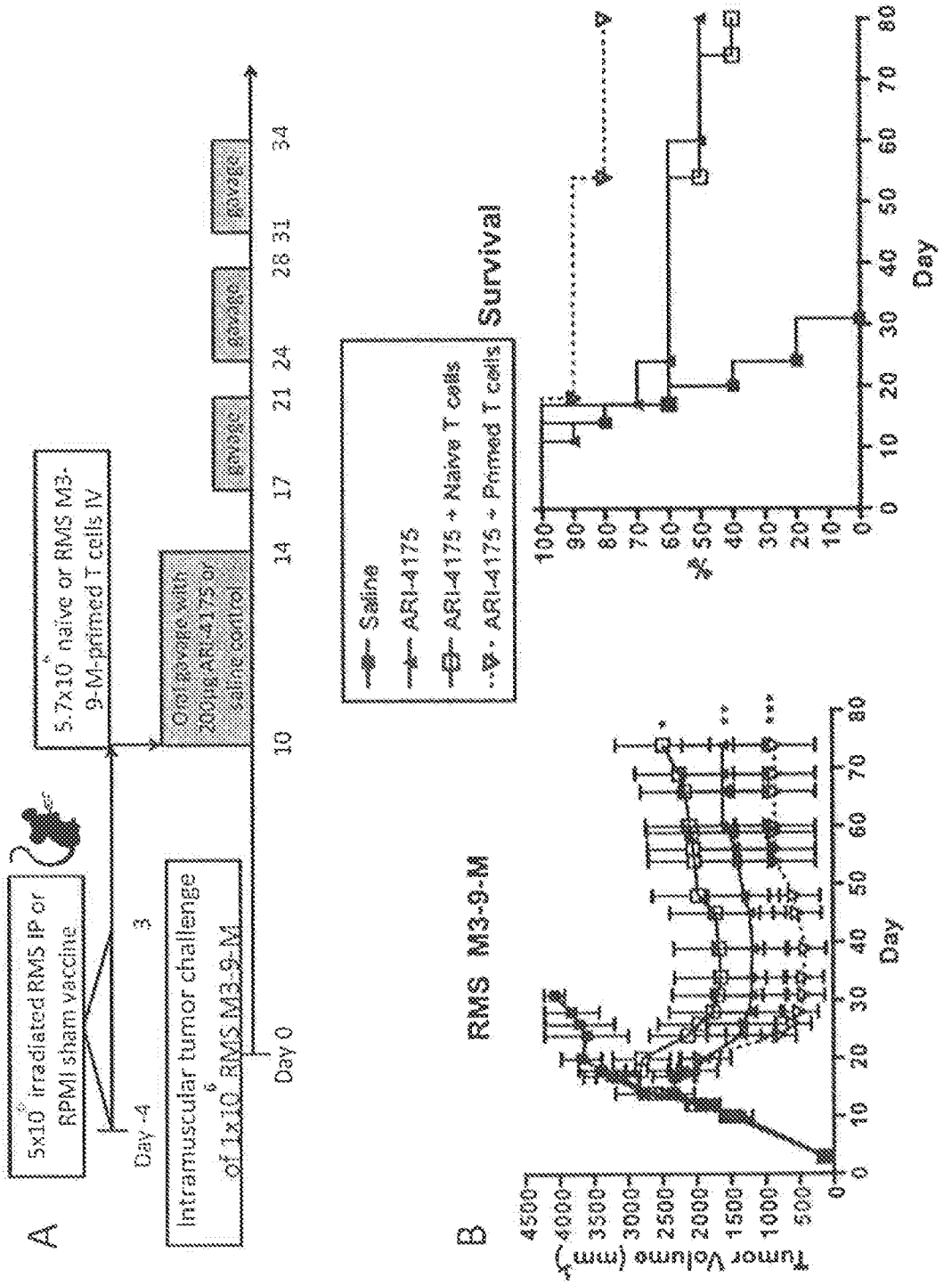


Figure 18

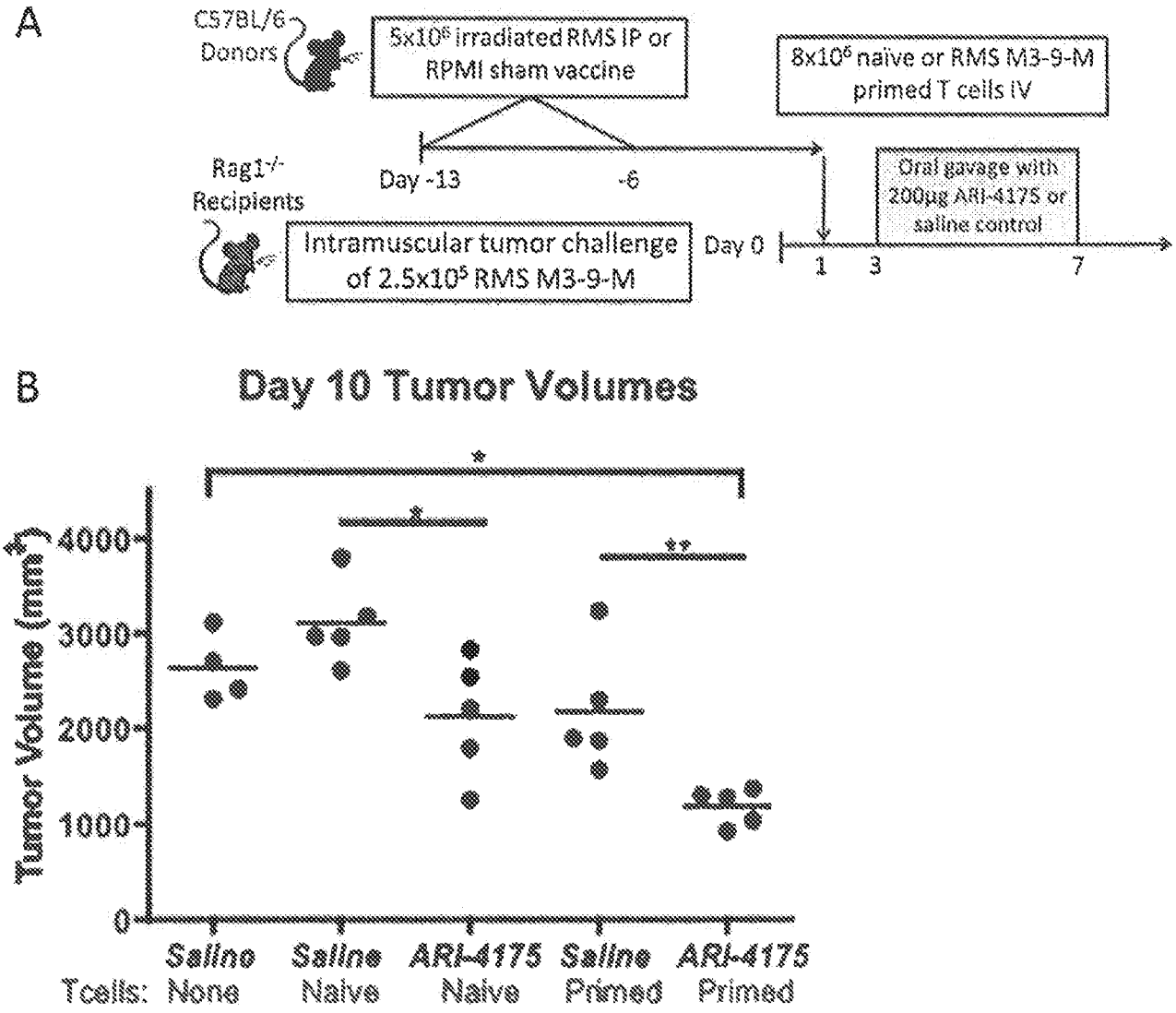

A
C57BL/6 Donors
5x10^6 irradiated RMS IP or RPMI sham vaccine
8x10^6 naive or RMS M3-9-M primed T cells IV
Rag1-/- Recipients
Day -13
-6
Oral gavage with 200µg ARI-4175 or saline control
Intramuscular tumor challenge of 2.5x10^5 RMS M3-9-M
Day 0
1
3
7
B
Day 10 Tumor Volumes
Tumor Volume (mm^3)
4000
3000
2000
1000
0
Saline
Saline
ARI-4175
Saline
ARI-4175
Tcells: None
Naive
Naive
Primed
Primed

Figure 19 A & B

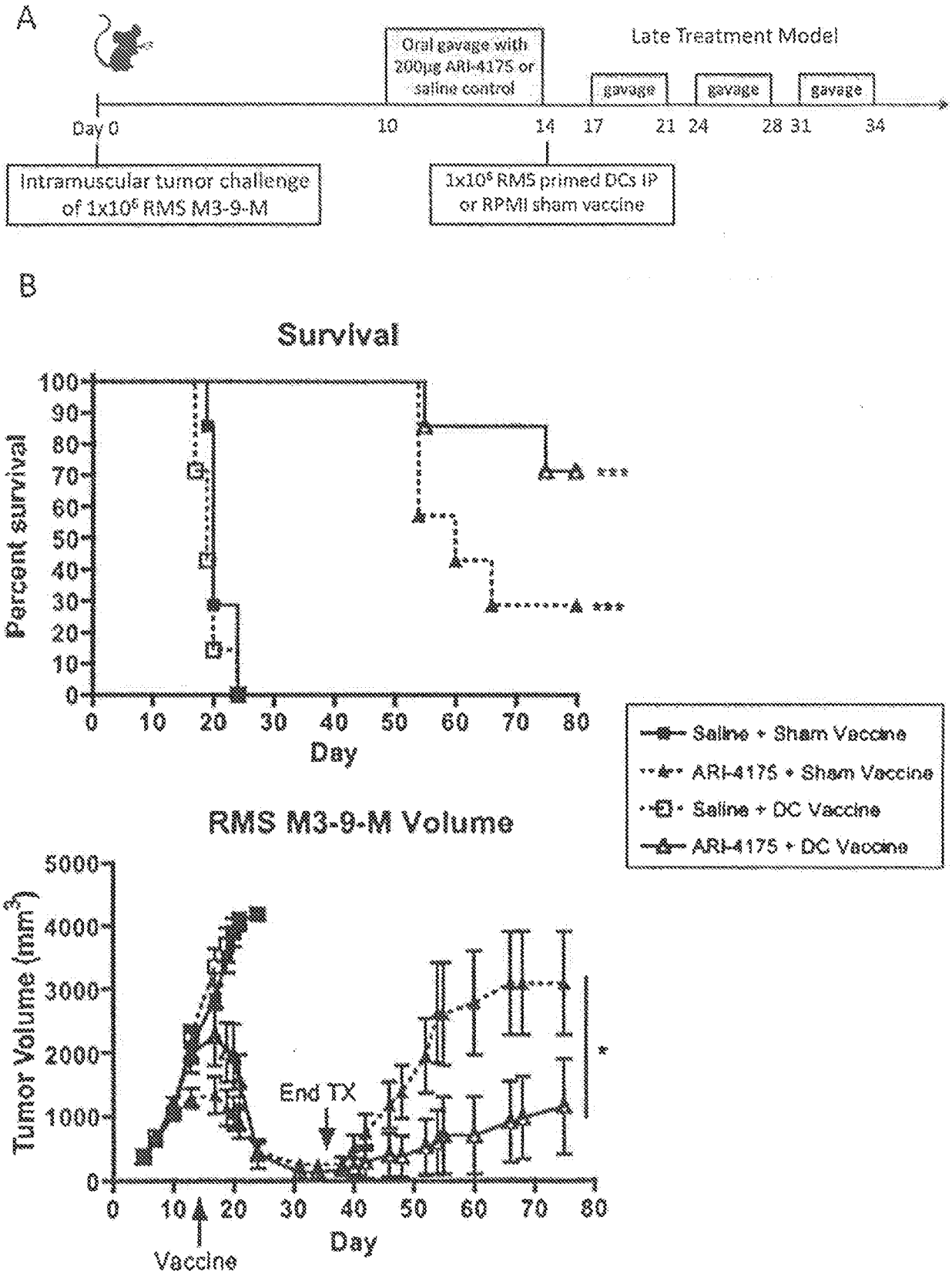
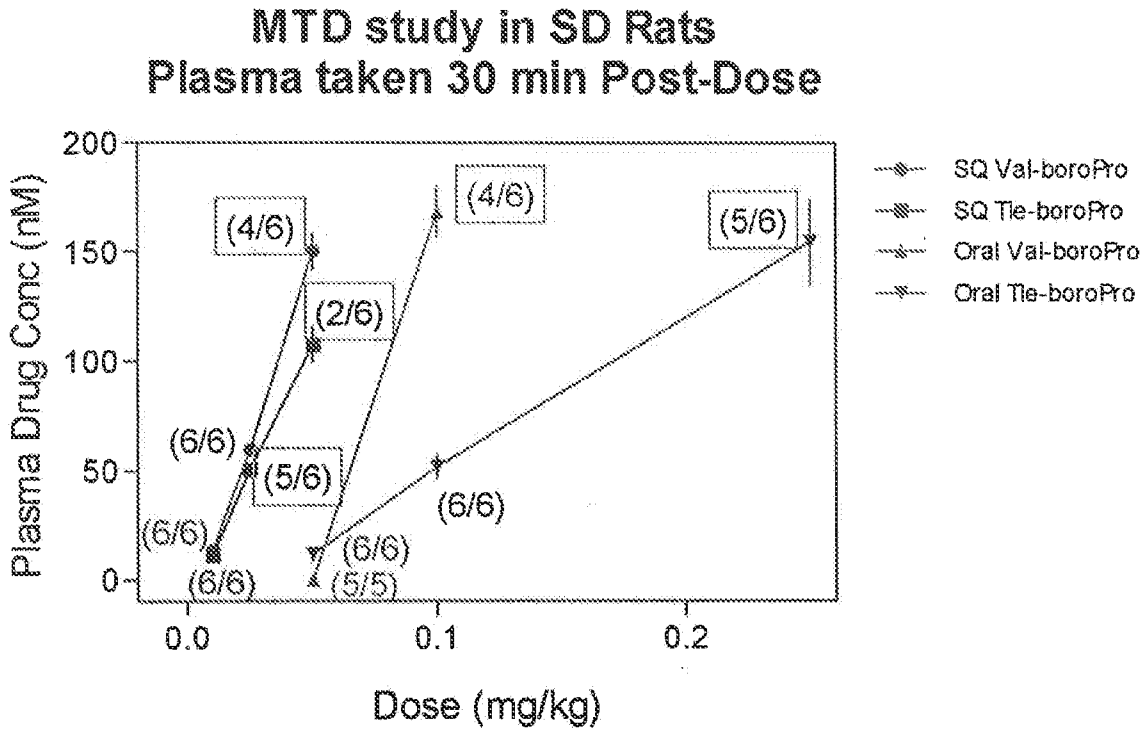

A
Late Treatment Model
Oral gavage with 200µg ARI-4175 or saline control
gavage
gavage
gavage
Day 0
10
14
17
21
24
28
31
34
Intramuscular tumor challenge of 1x10⁶ RMS M3-9-M
1x10⁶ RMS primed DCs IP or RPMI sham vaccine
B
Survival
Percent survival
Day
***
***
Saline + Sham Vaccine
ARI-4175 + Sham Vaccine
Saline + DC Vaccine
ARI-4175 + DC Vaccine
RMS M3-9-M Volume
Tumor Volume (mm³)
End TX
Vaccine
Day
*

Figure 20



Survival (number surviving/total per group) listed for each experiment.

Drug Concentration (nM)				
Dose (mg/kg)	Val-bPro (SQ)	Tle-bPro (SQ)	Val-bPro (PO)	Tle-bPro (PO)
0.01	13.67 (0.64)	11.11 (0.19)		
0.025	59.58 (2.25)	**50.63 (1.41)**		
0.05	**150.54 (8.49)**	**107.80 (8.41)**	0.45 (0.56)	11.90 (1.82)
0.1			**168.44 (11.41)**	51.87 (5.30)
0.25				**154.74 (19.76)**

Standard Error in Parentheses.

Figure 21

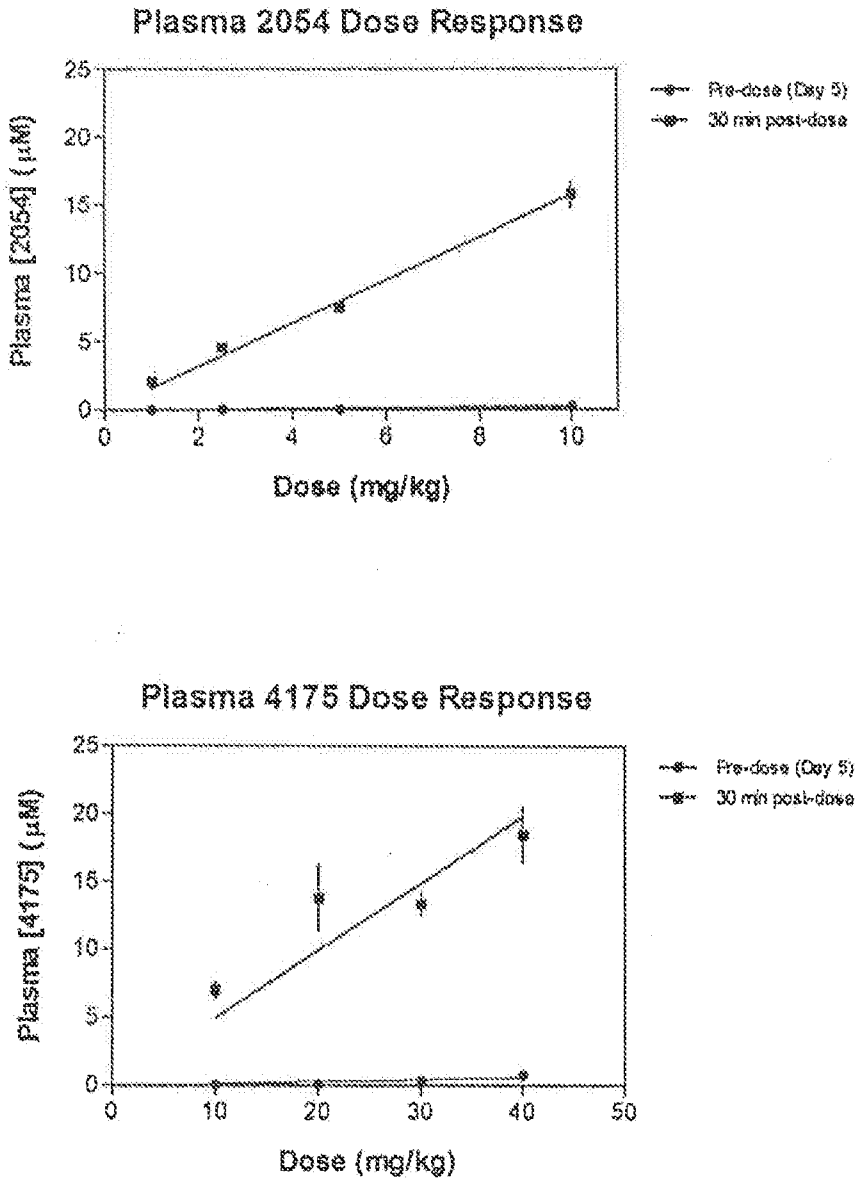
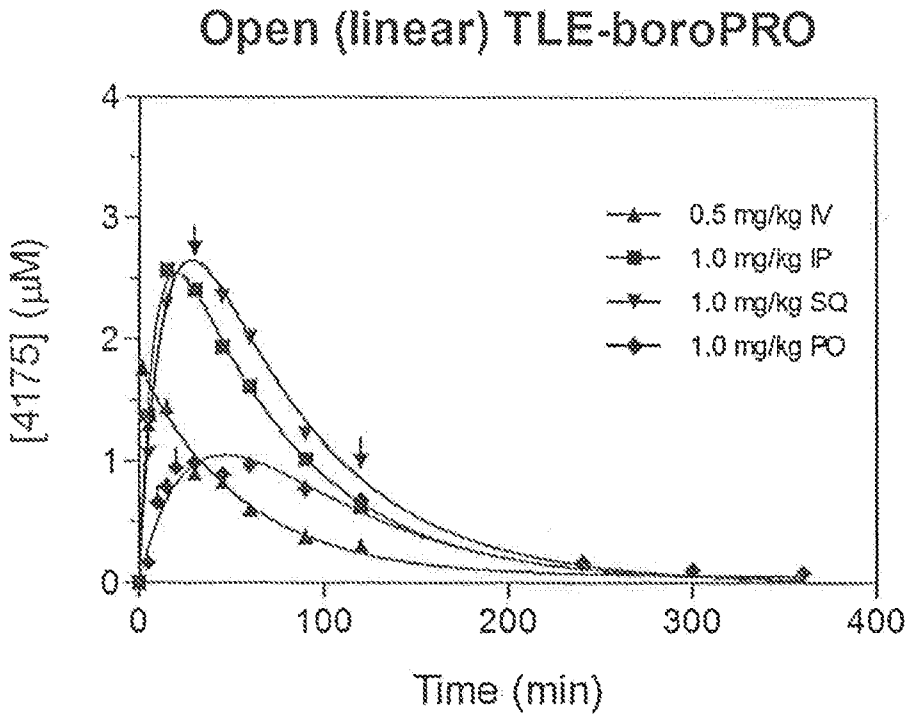

Plasma 2054 Dose Response
Plasma [2054] (μM)
25
20
15
10
5
0
0
2
4
6
8
10
Dose (mg/kg)
Pre-dose (Day 5)
30 min post-dose
Plasma 4175 Dose Response
Plasma [4175] (μM)
25
20
15
10
5
0
0
10
20
30
40
50
Dose (mg/kg)
Pre-dose (Day 5)
30 min post-dose

Figure 22


Open (linear) TLE-boroPRO
[4175] (μM)
Time (min)
0.5 mg/kg IV
1.0 mg/kg IP
1.0 mg/kg SQ
1.0 mg/kg PO
0
1
2
3
4
0
100
200
300
400

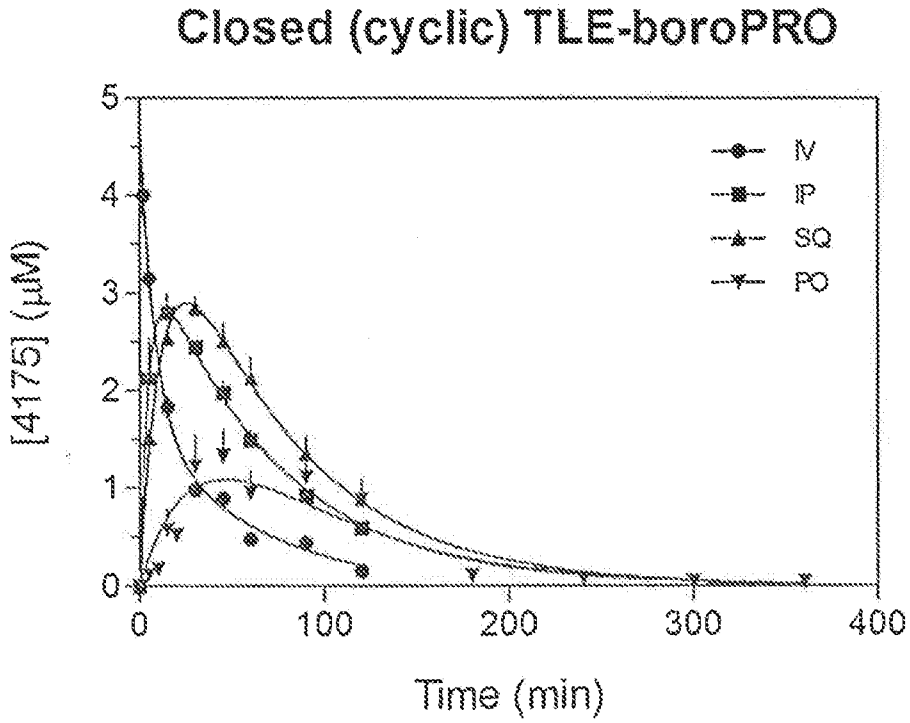
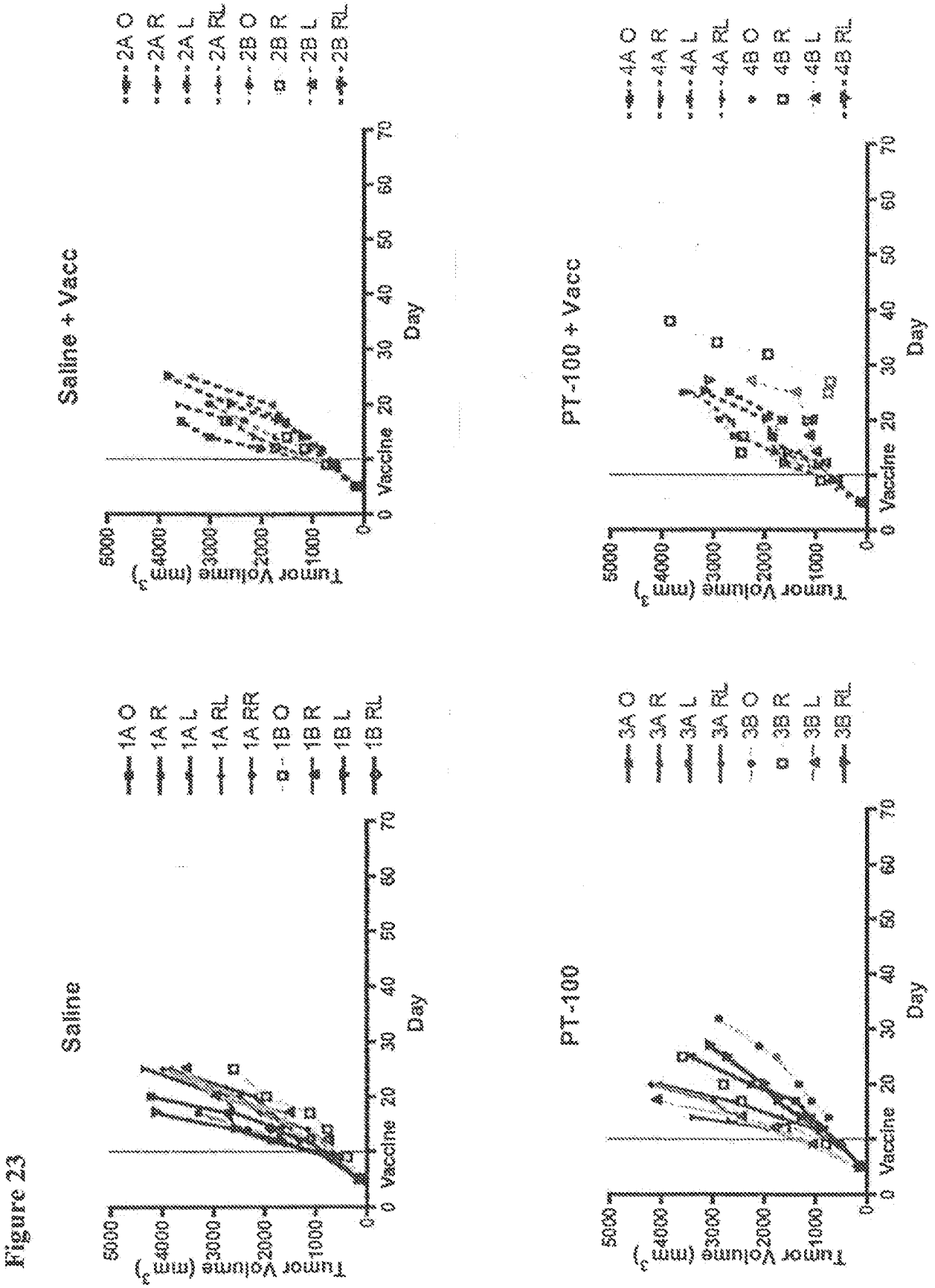

Closed (cyclic) TLE-boroPRO
[4175] (μM)
Time (min)
IV
IP
SQ
PO
0
1
2
3
4
5
0
100
200
300
400

Figure 23


Saline
1A O
1A R
1A L
1A RL
1A RR
1B O
1B R
1B L
1B RL
Tumor Volume (mm3)
Day
Vaccine
Saline + Vacc
2A O
2A R
2A L
2A RL
2B O
2B R
2B L
2B RL
PT-100
3A O
3A R
3A L
3A RL
3B O
3B R
3B L
3B RL
PT-100 + Vacc
4A O
4A R
4A L
4A RL
4B O
4B R
4B L
4B RL

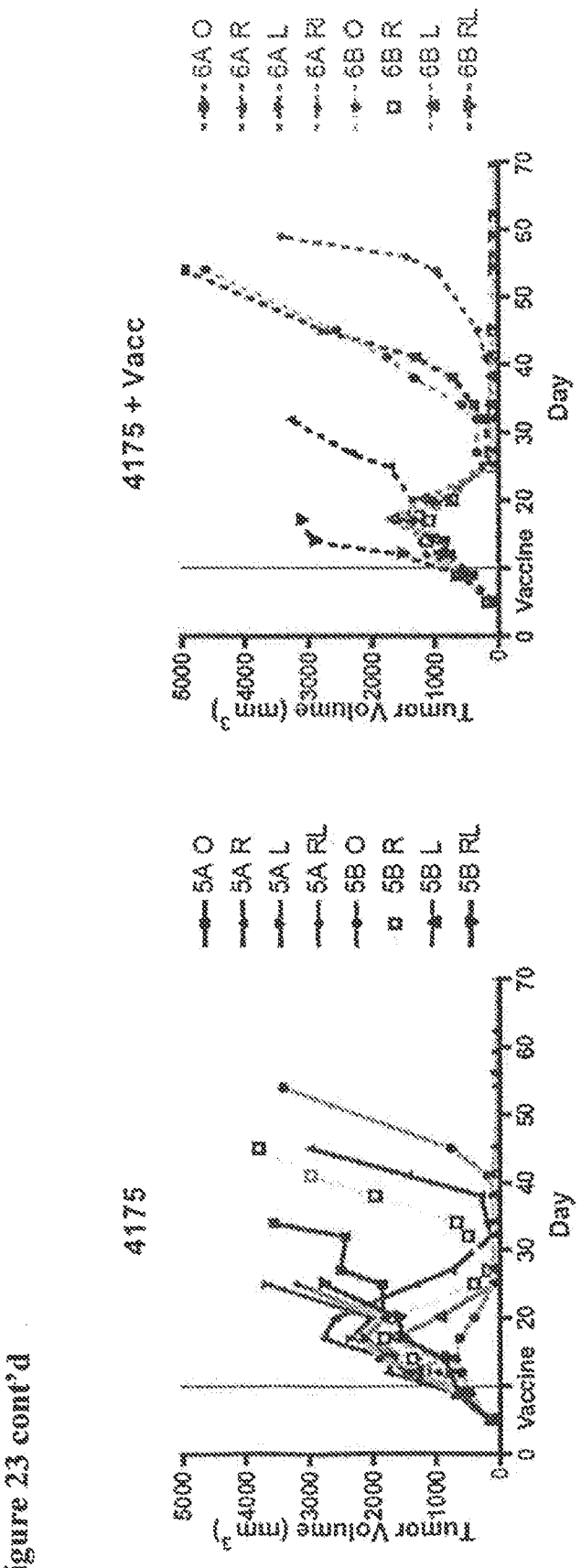


Figure 23 cont'd

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65236

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/07 (2012.01)
USPC - 435/344
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 435/344

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/7.23 (text search, see terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase; PubWEST(PGPB,USPT,EPAB,JPAB); Google Scholar (text search, see terms below)
Search Terms: Inhibit*, antagoniz*, reduc*, DPP*, DASH serine, CD26*, CXCL1, MGSA*, FSP, GRO1, Groalpha, NAP-3, GCSF, G-CSF, CSF3, increas*, induc*, enhanc*, DPP8, DP8, DPP9, DP9, Pro-boroPro, Pro-boroAla, Ala-boroPro

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/123686 A2 (MCLEAN et al.) 01 November 2007 (01.11.2007); Figures 2A, 2B, 2C, 2D, 6B, (pg 1, ln 31 - pg 2, ln 5), (pg 9, ln 7-8), (pg 23, ln 11-13), (pg 55, ln 25-26), (pg 138, ln 4-9), (pg 141, ln 11-18)	1-3
X	US 2009/0124559 A1 (BACHOVCHIN et al.) 14 May 2009 (14.05.2009); paras [0002], [0021], [0324], (Example 3; paras 0839-0840] with attached data table)	1, 2

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2013 (21.02.2013)	27 MAR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65236

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-31, 35-37, 41-46, 50-51 and 58-92
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-3, directed to a method of treating cancer, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases

Group II, Claims 32-34, directed to a method of increasing antitumor immunity in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases

----------Please see Extra Sheet----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65236

Continuation of Box III: Observations where unity of invention is lacking

Group III, Claims 38-40, directed to a method of stimulating or enhancing an immune response in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases

Group IV, Claims 47-49, directed to a method of treating a condition characterized by abnormal cell proliferation, comprising administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases

Group V, Claims 52-54, directed to a method of method of increasing cytokine and/or chemokine production in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases

Group VI, Claims 55-57, directed to a method of stimulating or enhancing production of T -cells in a mammal, comprising administering to a mammal in need thereof an effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases, wherein said T ?cells recognize an antigen on a malignant cell.

The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2 they lack the same or corresponding technical features for the following reasons:

Group I does not include a method of increasing antitumor immunity in a mammal as required by group II or a method of stimulating/enhancing an immune response in a mammal as required by group III or a method of treating a condition characterized by abnormal cell proliferation as required by group IV or a method of method of increasing cytokine and/or chemokine production in mammal as required by group V or a method of stimulating or enhancing production of T -cells in a mammal as required by group VI

Group II does not include a method of increasing antitumor immunity in a mammal as required by group I or a method of stimulating/enhancing an immune response in a mammal as required by group III or a method of treating a condition characterized by abnormal cell proliferation as required by group IV or a method of method of increasing cytokine and/or chemokine production in a mammal as required by group V or a method of stimulating or enhancing production of T -cells in a mammal as required by group VI

Group III does not include a method of increasing antitumor immunity in a mammal as required by group I or a method of increasing antitumor immunity in a mammal as required by group II or a method of treating a condition characterized by abnormal cell proliferation as required by group IV or a method of method of increasing cytokine and/or chemokine production in a mammal as required by group V or a method of stimulating or enhancing production of T -cells in a mammal as required by group VI

Group IV does not include a method of increasing antitumor immunity in a mammal as required by group I or a method of increasing antitumor immunity in a mammal as required by group II or a method of stimulating/enhancing an immune response in a mammal as required by group III or a method of method of increasing cytokine and/or chemokine production in a mammal as required by group V or a method of stimulating or enhancing production of T -cells in a mammal as required by group VI

Group V does not include a method of increasing antitumor immunity in a mammal as required by group I or a method of increasing antitumor immunity in a mammal as required by group II or a method of stimulating/enhancing an immune response in a mammal as required by group III or a method of treating a condition characterized by abnormal cell proliferation as required by group IV or a method of stimulating or enhancing production of T -cells in a mammal as required by group VI

Group VI does not include a method of increasing antitumor immunity in a mammal as required by group I or a method of increasing antitumor immunity in a mammal as required by group II or a method of stimulating/enhancing an immune response in a mammal as required by group III or a method of treating a condition characterized by abnormal cell proliferation as required by group IV or a method of method of increasing cytokine and/or chemokine production in a mammal as required by group V

The common technical feature of administering to a mammal in need thereof a therapeutically effective amount of a compound that inhibits a plurality of mammalian DASH serine proteases of groups I-VI is disclosed by US 2009/0124559 A1 to Bachovchin et al. (hereinafter 'Bachovchin'), 14 May 2009 (14.05.2009)

Bachovchin discloses group I invention, a method of treating cancer (para [0021]), comprising administering (para [0324]) to a mammal in need thereof (para [0002]) a therapeutically effective amount (para [0324]) of a compound that inhibits a plurality of mammalian DASH serine proteases (i.e., DPPIV, DPP8 (DP8), DPP9 (DP9); see instant invention page 15, Table 1) (para [0012]-[0013]; Example 3; paras [0839]-[0840] with attached data table). Groups I-VI therefore lack unity under PCT Rule 13 because they do not share a corresponding special technical feature.

Note: Claims 4-31, 35-37, 41-46, 50-51 and 58-92 determined unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

(19)中华人民共和国国家知识产权局

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权利要求书5页 说明书15页 附图26页

(54)发明名称

用于树突状细胞癌症疫苗的小分子增强剂

(57)摘要

公开了治疗癌症的方法，所述方法包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。还公开了哺乳动物中(a)增加抗肿瘤免疫力、(b)刺激或增强免疫应答、(c)治疗特征在于异常细胞增殖的状况、(d)增加细胞因子和/或趋化因子产生、或(e)刺激或增强T细胞产生的方法，所述方法包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。例如，抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物可以是叔丁基Gly-boroPro。

CN 104039952 A

1. 治疗癌症的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

2. 权利要求1的方法，其中所述化合物不是Val-boroPro。

3. 权利要求1或2的方法，其中所述化合物诱导选自GCSF和CXCL1的细胞因子的产生。

4. 权利要求1、2或3的方法，其中所述癌症选自基底细胞癌、胆道癌、膀胱癌、骨癌、脑癌、乳腺癌、宫颈癌、绒毛膜癌、CNS癌、结肠和直肠癌、结缔组织癌症、消化系统癌、子宫内膜癌、食管癌、眼癌、头颈部癌、胃癌、上皮内肿瘤、肾癌、喉癌、白血病、急性髓细胞白血病、急性淋巴性白血病、慢性髓细胞白血病、慢性淋巴性白血病、肝癌、小细胞肺癌、非小细胞肺癌、淋巴瘤、霍奇金氏淋巴瘤、非霍奇金氏淋巴瘤、黑色素瘤、骨髓瘤、神经母细胞瘤、口腔癌、卵巢癌、胰腺癌、前列腺癌、视网膜母细胞瘤、横纹肌肉瘤、直肠癌、肾癌、呼吸系统癌、肉瘤、皮肤癌、胃癌、睾丸癌、甲状腺癌、子宫癌和泌尿系统癌。

5. 权利要求1、2或3的方法，其中所述癌症选自前列腺癌、结肠直肠癌、多发性骨髓瘤和非小细胞肺癌。

6. 权利要求1、2或3的方法，其中所述癌症选自肺癌、结肠直肠癌、乳腺癌、胰腺癌和前列腺癌。

7. 权利要求1、2或3的方法，其中所述癌症是肺癌。

8. 权利要求7的方法，其中所述癌症是非小细胞肺癌。

9. 权利要求1、2或3的方法，其中所述癌症是结肠直肠癌。

10. 权利要求1、2或3的方法，其中所述癌症是乳腺癌。

11. 权利要求1、2或3的方法，其中所述癌症是胰腺癌。

12. 权利要求1、2或3的方法，其中所述癌症是前列腺癌。

13. 前述权利要求中任一项的方法，其中所述癌症是转移性的。

14. 前述权利要求中任一项的方法，进一步包括向所述哺乳动物共同施用治疗有效量的肿瘤引发的T细胞。

15. 权利要求14的方法，其中在所述施用所述化合物之前施用所述肿瘤引发的T细胞。

16. 权利要求14的方法，其中在所述施用所述化合物之后施用所述肿瘤引发的T细胞。

17. 权利要求14的方法，其中在所述施用所述化合物同时施用所述肿瘤引发的T细胞。

18. 权利要求1-13中任一项的方法，进一步包括向所述哺乳动物共同施用治疗有效量的口服活性肿瘤抗原。

19. 权利要求1-13中任一项的方法，进一步包括向所述哺乳动物共同施用治疗有效量的树突状细胞疫苗。

20. 前述权利要求中任一项的方法，进一步包括施用佐剂。

21. 权利要求1-13中任一项的方法，进一步包括用选自外科手术、放射和化疗的第二种疗法治疗所述哺乳动物。

22. 权利要求21的方法，其中所述第二种疗法是外科手术。

23. 权利要求21的方法，其中所述第二种疗法是放射。

24. 权利要求21的方法，其中所述第二种疗法是化疗。

25. 权利要求24的方法，其中所述化疗选自易普利姆玛、维罗非尼、GDC-0879、

PLX-4720、阿地白介素、天冬酰胺酶、硫酸博来霉素、卡铂、苯丁酸氮芥、顺铂、克拉屈滨、环磷酰胺、阿糖胞苷、达卡巴嗪、更生霉素、盐酸柔红霉素、多西他赛、多柔比星、盐酸多柔比星、盐酸表柔比星、依托泊苷、磷酸依托泊苷、氟尿苷、氟达拉滨、氟尿嘧啶、吉西他滨、盐酸吉西他滨、羟基脲、盐酸伊达比星、异环磷酰胺、干扰素类、干扰素 α 2a、干扰素 α 2b、干扰素 α n3、干扰素 α 1b、白介素、伊立替康、盐酸氮芥、美法仑、巯嘌呤、甲氨蝶呤、甲氨蝶呤钠、丝裂霉素、米托蒽醌、紫杉醇、培门冬酶、喷司他丁、泼尼松、卟吩姆钠、盐酸丙卡巴肼、紫杉酚、泰索帝、替尼泊苷、盐酸托泊替康、硫酸长春碱、硫酸长春新碱和酒石酸长春瑞滨。

26. 权利要求 24 的方法，其中所述化疗选自硫酸博来霉素、卡铂、顺铂、多西他赛、多柔比星、盐酸多柔比星、氟尿嘧啶、吉西他滨、盐酸吉西他滨、甲氨蝶呤、甲氨蝶呤钠、紫杉醇、紫杉酚、泰索帝、硫酸长春碱和硫酸长春新碱。

27. 权利要求 24 的方法，其中所述化疗是二肽基肽酶 IV 抑制剂。

28. 权利要求 24 的方法，其中所述化疗是 FAP 活化的化疗剂、FAP 活化的二肽基肽酶 IV 抑制剂或 FAP 活化的蛋白酶体抑制剂。

29. 权利要求 24 的方法，其中所述化疗是 FAP 活化的蛋白酶体抑制剂。

30. 权利要求 24 的方法，其中所述化疗是抗体。

31. 权利要求 30 的方法，其中所述抗体选自曲妥珠单抗、西妥昔单抗、贝伐单抗和利妥昔单抗。

32. 增加哺乳动物中的抗肿瘤免疫力的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

33. 权利要求 32 的方法，其中所述化合物不是 Val-boroPro。

34. 权利要求 32 或 33 的方法，其中所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

35. 权利要求 32、33 或 34 的方法，其中针对选自以下的肿瘤的所述抗肿瘤免疫力得到增加：肺肿瘤、淋巴瘤、乳腺肿瘤、结肠直肠肿瘤、甲状腺肿瘤、子宫肿瘤、胰腺肿瘤、前列腺肿瘤、皮肤肿瘤、肾肿瘤、肝肿瘤和脑肿瘤。

36. 权利要求 32、33 或 34 的方法，其中针对选自以下的肿瘤的所述抗肿瘤免疫力得到增加：肺肿瘤、乳腺肿瘤、结肠直肠肿瘤、胰腺肿瘤和前列腺肿瘤。

37. 权利要求 32-36 中任一项的方法，其中所述抗肿瘤免疫力包含抗体依赖性细胞介导的细胞毒性。

38. 刺激或增强哺乳动物中的免疫应答的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

39. 权利要求 38 的方法，其中所述化合物不是 Val-boroPro。

40. 权利要求 38 或 39 的方法，其中所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

41. 权利要求 38、39 或 40 的方法，其中所述免疫应答得到刺激。

42. 权利要求 38、39 或 40 的方法，其中所述免疫应答得到增强。

43. 权利要求 38-42 中任一项的方法，其中所述免疫应答包含抗体依赖性细胞介导的细胞毒性。

44. 权利要求 38-43 中任一项的方法，其中所述哺乳动物具有癌症或处于发生癌症的

风险中。

45. 权利要求 38-43 中任一项的方法，其中所述哺乳动物在癌症的缓解中。

46. 权利要求 38-43 中任一项的方法，其中所述哺乳动物具有难治性或耐受性癌症。

47. 治疗特征在于异常细胞增殖的状况的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

48. 权利要求 47 的方法，其中所述化合物不是 Val-boroPro。

49. 权利要求 47 或 48 的方法，其中所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

50. 权利要求 47、48 或 49 的方法，其中所述异常细胞增殖是癌症、血管增生性病症或纤维化病症。

51. 权利要求 47、48 或 49 的方法，其中所述异常细胞增殖是异常血管发生。

52. 增加哺乳动物中的细胞因子和 / 或趋化因子产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

53. 权利要求 52 的方法，其中所述化合物不是 Val-boroPro。

54. 权利要求 52 或 53 的方法，其中所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

55. 刺激或增强哺乳动物中的 T 细胞产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物，其中所述 T 细胞识别恶性细胞上的抗原。

56. 权利要求 55 的方法，其中所述化合物不是 Val-boroPro。

57. 权利要求 55 或 56 的方法，其中所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

58. 权利要求 55、56 或 57 的方法，其中所述 T 细胞产生得到刺激。

59. 权利要求 55、56 或 57 的方法，其中所述 T 细胞产生得到增强。

60. 权利要求 55-59 中任一项的方法，其中所述恶性细胞是癌、肉瘤、白血病、淋巴瘤或骨髓瘤。

61. 前述权利要求中任一项的方法，其中所述哺乳动物是灵长类动物、犬、马、猫或牛。

62. 前述权利要求中任一项的方法，其中所述哺乳动物是人。

63. 前述权利要求中任一项的方法，其中口服或肠胃外施用所述化合物。

64. 权利要求 63 的方法，其中肠胃外施用所述化合物。

65. 权利要求 63 的方法，其中口服施用所述化合物。

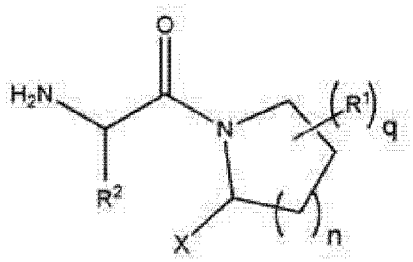
66. 权利要求 65 的方法，其中以固体剂型施用所述化合物。

67. 权利要求 66 的方法，其中所述固体剂型是片剂、胶囊或丸剂。

68. 权利要求 66 的方法，其中所述固体剂型是片剂。

69. 前述权利要求中任一项的方法，其中以足以刺激免疫系统而没有剂量限制性毒性的量施用所述化合物。

70. 权利要求 1-69 中任一项的方法，其中所述化合物由式 I 代表：



式 I

其中：

X 是 $B(Y^1)(Y^2)$ 或 CN；

Y^1 和 Y^2 独立地为 OH，或与它们连接的硼原子一起代表可水解为硼酸的基团，或与它们连接的硼原子一起形成可水解为硼酸的 5 至 8 元环；

R^1 选自卤素、低级烷基、低级烯基、低级炔基、羰基、羧基、酯、甲酸酯、酮、硫代羰基、硫酯、硫代乙酸酯、硫代甲酸酯、氨基、酰基氨基、酰胺基、硝基、硫酸酯、磺酸酯、磺酰胺基、$-(CH_2)_m-R_7$、$-(CH_2)_m-OH$、$-(CH_2)_m-O-$ 低级烷基、$-(CH_2)_m-O-$ 低级烯基、$-(CH_2)_n-O-(CH_2)_m-R_7$、$-(CH_2)_m-SH$、$-(CH_2)_m-S-$ 低级烷基、$-(CH_2)_m-S-$ 低级烯基、或 $-(CH_2)_n-S-(CH_2)_m-R_7$、叠氮基、氰基、异氰酸根合、硫氰酸根合、异硫氰酸根合、氰氧基、  、或  ；

R_7 代表取代或未取代的芳基、芳烷基、环烷基、环烯基或杂环；

R_8 独立地代表氢、$-CH_3$ 或 $-(CH_2)_n-CH_3$；

m 是 0、1、2、3、4、5、或 6；

R^2 是选自正丙基、C_4-C_8 烷基、C_2-C_8 烯基、C_2-C_8 炔基、C_3-C_8 环烷基、C_2-C_7 杂环基、芳基、杂芳基、芳烷基、杂芳烷基和天然存在的疏水性氨基酸的侧链的疏水基团；

n 是 0、1 或 2；且

q 是 0、1、2、3 或 4。

71. 权利要求 70 的方法，其中 q 是 0、1 或 2。

72. 权利要求 70 的方法，其中 q 是 0。

73. 权利要求 70-72 中任一项的方法，其中 n 是 0。

74. 权利要求 70-72 中任一项的方法，其中 n 是 1。

75. 权利要求 70-72 中任一项的方法，其中 n 是 2。

76. 权利要求 70-75 中任一项的方法，其中 X 是 $B(Y^1)(Y^2)$。

77. 权利要求 70-75 中任一项的方法，其中 X 是 $B(OH)_2$。

78. 权利要求 70 的方法，其中 n 是 1；q 是 0；且 X 是 $B(OH)_2$。

79. 权利要求 70-78 中任一项的方法，其中 R^2 选自叔丁基、异丁基、戊基、环己基、苄基或萘基。

80. 权利要求 70-78 中任一项的方法，其中 R^2 选自叔丁基、异丁基或戊基。

81. 权利要求 70-78 中任一项的方法，其中 R^2 是叔丁基。

82. 权利要求 70-78 中任一项的方法，其中 R^2 是天然存在的疏水性氨基酸的侧链。

83. 权利要求 70-78 中任一项的方法，其中 R^2 是亮氨酸、异亮氨酸、叔亮氨酸、苯丙氨酸

或色氨酸的侧链。

84. 权利要求 70 的方法，其中式 I 的化合物是叔丁基 Gly-boroPro。

85. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 L。

86. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 D。

87. 权利要求 70-84 中任一项的方法，其中在携带 R^2 的碳处的立体化学构型是 L。

88. 权利要求 70-84 中任一项的方法，其中在携带 R^2 的碳处的立体化学构型是 D。

89. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 L；且在携带 R^2 的碳处的立体化学构型是 L。

90. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 L；且在携带 R^2 的碳处的立体化学构型是 D。

91. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 D；且在携带 R^2 的碳处的立体化学构型是 L。

92. 权利要求 70-84 中任一项的方法，其中在携带 X 的碳处的立体化学构型是 D；且在携带 R^2 的碳处的立体化学构型是 D。

用于树突状细胞癌症疫苗的小分子增强剂

[0001] *相关申请*

本申请要求2011年11月22日提交的美国临时专利申请系列号61/562,497的优先权权益。

[0002] *发明背景*

癌症是美国的第二大死亡原因。批准的抗癌药剂，化疗药剂和靶向药剂两者，都受毒性限制，对实体瘤最终无效，所述实体瘤例如：肺癌、结肠直肠癌、乳腺癌、胰腺癌和前列腺癌，其占癌症死亡的85％以上。使用机体免疫系统杀死肿瘤（其失败使得癌症出现），长久以来已经是癌症研究的目标。Val-boroPro，也称为PT-100或talabostat，是一种二肽硼酸，其通过免疫活化在小鼠中缩小肿瘤中显示显著效力。然而，在快速追踪III期临床试验(Fast Track Phase III clinical trials)中，其由于剂量限制毒性没有实现其目标。

[0003] 美国食品和药物管理局在2010年4月29日批准了用于前列腺癌的第一个癌症疫苗Provenge。Provenge是树突状细胞治疗(DCT)；有时被称为“癌症疫苗”的几种令人激动的新免疫治疗之一。通过增压免疫系统，此类疫苗在原则上可以发现并去除非常最后的癌细胞，无论它隐藏在哪里，从而排除疗程之后的唯一缓解。尽管现在证明了该概念，但癌症疫苗，包括除了Provenge以外的DCT，在临床试验中都未能实现所需效力，表明需要添加免疫刺激剂或佐剂。然而，在临床上需要较少毒性佐剂来开发这种方法。

[0004] *发明概述*

本发明的一个方面涉及治疗癌症的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

[0005] 本发明的另一个方面涉及增加哺乳动物中的抗肿瘤免疫力的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

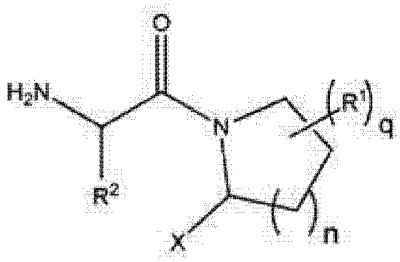
[0006] 本发明的另一个方面涉及刺激或增强哺乳动物中的免疫应答的方法，包括向有需要的哺乳动物施用有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

[0007] 本发明的又另一个方面涉及治疗特征在于异常细胞增殖的状况的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

[0008] 本发明的另一个方面涉及增加哺乳动物中的细胞因子和趋化因子产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

[0009] 本发明的另一个方面涉及刺激或增强哺乳动物中的T细胞产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物，其中所述T细胞识别恶性细胞上的抗原。

[0010] 在某些实施方案中，本发明涉及上述方法中的任一种，其中所述化合物由式I代表：



式 I

其中：

X 是 B(Y[1])(Y[2]) 或 CN；

Y[1] 和 Y[2] 独立地为 OH，或与它们连接的硼原子一起代表可水解为硼酸的基团，或与它们连接的硼原子一起形成可水解为硼酸的 5 至 8 元环；

R[1] 选自卤素、低级烷基、低级烯基、低级炔基、羰基、羧基、酯、甲酸酯、酮、硫代羰基、硫酯、硫代乙酸酯、硫代甲酸酯、氨基、酰基氨基、酰胺基、硝基、硫酸酯、磺酸酯、磺酰胺基、$-(CH_2)_m-R_7$、$-(CH_2)_m-OH$、$-(CH_2)_m-O-$ 低级烷基、$-(CH_2)_m-O-$ 低级烯基、$-(CH_2)_n-O-(CH_2)_m-R_7$、$-(CH_2)_m-SH$、$-(CH_2)_m-S-$ 低级烷基、$-(CH_2)_m-S-$ 低级烯基、或 $-(CH_2)_n-S-(CH_2)_m-R_7$、叠氮基、氰基、异氰酸根合、硫氰酸根合、异硫氰酸根合、氰氧基、  、或  ；

R_7 代表取代或未取代的芳基、芳烷基、环烷基、环烯基或杂环；

R_8 独立地代表氢、$-CH_3$ 或 $-(CH_2)_n-CH_3$；

M 是 0、1、2、3、4、5 或 6；

R^2 是选自正丙基、C_4-C_8 烷基、C_2-C_8 烯基、C_2-C_8 炔基、C_3-C_8 环烷基、C_2-C_7 杂环基、芳基、杂芳基、芳烷基、杂芳烷基、和天然存在的疏水性氨基酸的侧链的疏水基团；

n 是 0、1 或 2；且

q 是 0、1、2、3 或 4。

[0011] 在某些实施方案中，本发明涉及上述方法中的任一种，其中所述化合物是叔丁基 Gly-boroPro (*t*-butylGly-boroPro)。

[0012] *附图简述*

图 1 显示，Ala-boroPro (A-bP) 和 Val-boroPro (V-bP) 都抑制脯氨酰寡蛋白酶 (oligoprotease)，但 Ala-boroPro 不刺激免疫系统。Val-boroPro 是有效的免疫刺激剂。

[0013] 图 2 显示，PT-100 产生早期肿瘤的完全消退，但不产生已建立肿瘤的完全消退。

[0014] 图 3 显示，PT-100 + 树突状细胞 (DC) 疫苗产生已建立肿瘤的消退。

[0015] 图 4 显示，Val-boroPro（图中的 2054），但不是 Ala-boroPro（图中的 2054）刺激 BALB/c 小鼠中的 G-CSF 和 CXCL1/KC。G-CSF 和 CXL1/KC 是抗癌免疫增强活性的标记物。实验设置和结果在实施例 3 中讨论。

[0016] 图 5 显示，Ari-4175（图中的 4175-2）是细胞因子在体内的一种非常有效的诱导物。实验设置和结果在实施例 3 中讨论。

[0017] 图 6 显示，ARI-4175 (4175-2) 在诱导 G-CSF 和 CXCL1 细胞因子（其是抗癌免疫增强活性的标记物）时比 PT-100 (2054) 效力高得多。实验设置和结果在实施例 3 中讨论。

[0018] 图 7 显示，ARI-4175 在 M3-9-M RMS 模型中建立针对肿瘤再攻击的免疫力。实验设置和结果在实施例 4 中讨论。

[0019] 图 8 A 和 B 显示，ARI-4175 增加了 DC 疫苗在横纹肌肉瘤 (RMS) 模型中抑制已建立的 RMS 的生长并增加小鼠存活的效力。在第 0 天用 RMS 细胞肌内注射 C57BL/6 小鼠。如实施例 1 所述，小鼠在第 10 天接受单次皮下接种，随后每天管饲 10 mg/kg (5 天) 和 5 mg/kg (10 天) ARI-4175、1 mg/kg PT-100 (15 天) 或媒介物。

[0020] 图 9 显示实验 1 中所述的实验中个体小鼠中的肿瘤生长。

[0021] 图 10 显示，ARI-4175 (4175) 没有表现出针对结肠直肠癌细胞系的显著体外活性。当 4175 与西妥昔单抗 (CTX) 组合时，也没有任何活性。这是预料到的，因为 ARI-4175 和西妥昔单抗的抗肿瘤作用被认为是通过抗体依赖性细胞介导的细胞毒性 (ADCC) 介导的。

[0022] 图 11 显示，在取自用 ARI-4175 治疗的裸鼠的 NK 细胞上 CD16 表达上调。

[0023] 图 12 显示，在取自用 ARI-4175 治疗的裸鼠的 NK 细胞上 LAMP-1 (CD107) 表达上调。

[0024] 图 13 A 和 B 显示 ARI-4175 对结肠癌异种移植物的体内抑制。A 显示单独或与西妥昔单抗 (CTX) 组合的 ARI-4175 对 DLD1 异种移植物的抑制。B 显示单独或与西妥昔单抗 (CTX) 组合的 ARI-4175 对 HCT-116 的抑制。

[0025] 图 14 显示，来自用 ARI-4175 和 / 或西妥昔单抗 (CTX) 治疗的 C57BI/6 未携带肿瘤的小鼠的脾细胞针对 HCT116 肿瘤细胞的细胞毒性得到增强。

[0026] 图 15 显示，ARI-4175 诱导人 NK 细胞上的 CD69。显示的结果是用 1 天孵育后的来自两个健康供体的培养的人 PBL。

[0027] 图 16 显示，ARI-4175 与 PT-100 一样有效，但在高剂量的毒性较小。

[0028] 图 17 A 和 B 显示，作为肿瘤引发的 T 细胞转移的佐剂，ARI-4175 诱导晚期治疗 RMS M3-9-M 模型中的肿瘤消退。

[0029] 图 18 A 和 B 显示，Rag1-/- 受体中用 ARI-4175 和过继性 T 细胞转移联合治疗显著降低 RMS M3-9-M 体积。(A) 雌性 $Rag1^{-/-}$ 小鼠在肿瘤攻击后一天接受未引发的 (naïve) 或 RMS 引发的 T 细胞。(B) 到第 10 天，ARI-4175 治疗的小鼠与盐水治疗的小鼠相比具有显著较小的肿瘤 (未引发的 :n=5, p=0.0159 ;引发的 :n=5, p=0.0079)。

[0030] 肿瘤引发的 T 细胞与 ARI-4175 组合治疗的 $Rag1^{-/-}$ 小鼠具有总体最小的肿瘤。

[0031] 图 19 ARI-4175 是 DC 接种的有效的佐剂，如显著改善的存活和降低的 RMS M3-9-M 体积所证明。

[0032] 图 20 显示使用 SD 大鼠用给药后 30 分钟采集的数据的最大耐受剂量 (MTD) 研究的结果。

[0033] 图 21 显示用于 PT-100 和 ARI-4175 的剂量血浆应答曲线。

[0034] 图 22 显示 ARI-4175 的打开 (open) 相比于闭合 (closed) 形式的药代动力学数据。在酸性条件下，ARI-4175 以打开的、或线形形式存在 ；在中性或碱性条件下，闭合的、环化的形式是高度有利的。

[0035] 图 23 显示 RMS 模型中个体小鼠中的肿瘤生长。

[0036] *发明详述*

本发明的一个方面涉及治疗癌症的方法，包括向有其需要的哺乳动物施用治疗有效量

的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物。

[0037] 本发明的另一个方面涉及治疗癌症的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物，其中所述化合物不是Val-boroPro。

[0038] 本发明的另一个方面涉及前述方法中的任一种，其中所述化合物诱导选自GCSF和CXCL1的细胞因子的产生。

[0039] 本发明的另一个方面涉及前述方法中的任一种，其中所述癌症选自基底细胞癌、胆道癌、膀胱癌、骨癌、脑癌、乳腺癌、宫颈癌、绒毛膜癌、CNS癌、结肠和直肠癌、结缔组织癌症、消化系统癌、子宫内膜癌、食管癌、眼癌、头颈部癌、胃癌(gastric cancer)、上皮内肿瘤、肾癌(kidney cancer)、喉癌、白血病、急性髓细胞白血病、急性淋巴性白血病、慢性髓细胞白血病、慢性淋巴性白血病、肝癌、小细胞肺癌、非小细胞肺癌、淋巴瘤、霍奇金氏淋巴瘤、非霍奇金氏淋巴瘤、黑色素瘤、骨髓瘤、神经母细胞瘤、口腔癌、卵巢癌、胰腺癌、前列腺癌、视网膜母细胞瘤、横纹肌肉瘤、直肠癌、肾癌(renal cancer)、呼吸系统癌、肉瘤、皮肤癌、胃癌(stomach cancer)、睾丸癌、甲状腺癌、子宫癌、和泌尿系统癌。

[0040] 在其他实施方案中，所述癌症选自前列腺癌、结肠直肠癌、多发性骨髓瘤和非小细胞肺癌。

[0041] 在某些其他实施方案中，所述癌症选自肺癌、结肠直肠癌、乳腺癌、胰腺癌和前列腺癌。

[0042] 在一个实施方案中，所述癌症是肺癌。

[0043] 在另一个实施方案中，所述癌症是非小细胞肺癌。

[0044] 在又另一个实施方案中，所述癌症是结肠直肠癌。

[0045] 在某些实施方案中，所述癌症是乳腺癌。

[0046] 在某些其他实施方案中，所述癌症是胰腺癌。

[0047] 在另一个实施方案中，所述癌症是前列腺癌。

[0048] 在某些实施方案中，所述癌症是转移性的。

[0049] 本发明的另一个方面涉及上述方法中的任一种，进一步包括向哺乳动物共同施用治疗有效量的肿瘤引发的T细胞。

[0050] 在某些实施方案中，在施用化合物之前施用肿瘤引发的T细胞。

[0051] 在某些实施方案中，在施用化合物之后施用肿瘤引发的T细胞。

[0052] 在某些实施方案中，与施用化合物同时施用肿瘤引发的T细胞。

[0053] 本发明的另一个方面涉及上述方法中的任一种，进一步包括向哺乳动物共同施用治疗有效量的口服活性肿瘤抗原。

[0054] 本发明的又另一个方面涉及上述方法中的任一种，进一步包括向哺乳动物共同施用治疗有效量的树突状细胞疫苗。

[0055] 本发明的仍另一个方面涉及上述方法中的任一种，进一步包括施用佐剂。

[0056] 本发明的另一个方面涉及前述实施方案中的任一个，进一步包括用选自外科手术、放射和化疗的第二种疗法治疗哺乳动物。

[0057] 在一个实施方案中，第二种疗法是外科手术。

[0058] 在另一个实施方案中，第二种疗法是放射。

[0059] 在又另一个实施方案中，第二种疗法是化疗。

[0060] 在某些实施方案中，所述化疗选自易普利姆玛、维罗非尼、GDC-0879、PLX-4720、阿地白介素、天冬酰胺酶、硫酸博来霉素、卡铂、苯丁酸氮芥、顺铂、克拉屈滨、环磷酰胺、阿糖胞苷、达卡巴嗪、更生霉素、盐酸柔红霉素、多西他赛、多柔比星、盐酸多柔比星、盐酸表柔比星、依托泊苷、磷酸依托泊苷、氟尿苷、氟达拉滨、氟尿嘧啶、吉西他滨、盐酸吉西他滨、羟基脲、盐酸伊达比星、异环磷酰胺、干扰素类、干扰素 α 2a、干扰素 α 2b、干扰素 α n3、干扰素 α 1b、白介素、伊立替康、盐酸氮芥、美法仑、巯嘌呤 (mercatopurine)、甲氨蝶呤、甲氨蝶呤钠、丝裂霉素、米托蒽醌、紫杉醇、培门冬酶、喷司他丁、泼尼松、卟吩姆钠、盐酸丙卡巴肼 (procabazine hydrochloride)、紫杉酚、泰索帝、替尼泊苷、盐酸托泊替康、硫酸长春碱、硫酸长春新碱和酒石酸长春瑞滨。

[0061] 在某些实施方案中，所述化疗选自硫酸博来霉素、卡铂、顺铂、多西他赛、多柔比星、盐酸多柔比星、氟尿嘧啶、吉西他滨、盐酸吉西他滨、甲氨蝶呤、甲氨蝶呤钠、紫杉醇、紫杉酚、泰索帝、硫酸长春碱和硫酸长春新碱。

[0062] 在某些实施方案中，所述化疗是二肽基肽酶 IV 抑制剂。

[0063] 在某些其他实施方案中，所述化疗是 FAP 活化的化疗剂、FAP 活化的二肽基肽酶 IV 抑制剂或 FAP 活化的蛋白酶体抑制剂。

[0064] 在仍其他实施方案中，所述化疗是 FAP 活化的蛋白酶体抑制剂。

[0065] 在某些实施方案中，所述化疗是抗体。

[0066] 在某些其他实施方案中，所述抗体选自曲妥珠单抗、西妥昔单抗、贝伐单抗和利妥昔单抗。

[0067] 本发明的一个方面涉及增加哺乳动物中的抗肿瘤免疫力的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

[0068] 在某些实施方案中，所述化合物不是 Val-boroPro。

[0069] 在某些其他实施方案中，所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

[0070] 在又其他实施方案中，针对选自以下的肿瘤的抗肿瘤免疫力得到增加：肺肿瘤、淋巴瘤、乳腺肿瘤、结肠直肠肿瘤、甲状腺肿瘤、子宫肿瘤、胰腺肿瘤、前列腺肿瘤、皮肤肿瘤、肾肿瘤、肝肿瘤和脑肿瘤。

[0071] 在其他实施方案中，针对选自以下的肿瘤的抗肿瘤免疫力得到增加：肺肿瘤、乳腺肿瘤、结肠直肠肿瘤、胰腺肿瘤和前列腺肿瘤。

[0072] 在某些其他实施方案中，所述抗肿瘤免疫力包含抗体依赖性细胞介导的细胞毒性。

[0073] 本发明的另一个方面涉及刺激或增强哺乳动物中的免疫应答的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

[0074] 在某些实施方案中，所述化合物不是 Val-boroPro。

[0075] 在某些其他实施方案中，所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

[0076] 在仍其他实施方案中，所述免疫应答得到刺激。

[0077] 在仍又进一步实施方案中，所述免疫应答得到增强。

[0078] 在某些实施方案中，所述免疫应答包含抗体依赖性细胞介导的细胞毒性。

[0079] 在某些其他实施方案中，所述哺乳动物具有癌症或处于发生癌症的风险中。

[0080] 在仍其他实施方案中，所述哺乳动物在癌症的缓解中。

[0081] 在仍又进一步实施方案中，所述哺乳动物具有难治性或耐受性癌症。

[0082] 本发明的另一个方面涉及治疗特征在于异常细胞增殖的状况的方法，包括向有其需要的哺乳动物施用治疗有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

[0083] 在某些实施方案中，所述化合物不是 Val-boroPro。

[0084] 在某些实施方案中，所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

[0085] 在某些实施方案中，所述异常细胞增殖是癌症、血管增生性病症或纤维化病症。

[0086] 在某些实施方案中，所述异常细胞增殖是异常血管发生。

[0087] 本发明的另一个方面涉及增加哺乳动物中的细胞因子和 / 或趋化因子产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物。

[0088] 在某些实施方案中，所述化合物不是 Val-boroPro。

[0089] 在某些其他实施方案中，所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

[0090] 本发明的另一个方面涉及刺激或增强哺乳动物中的 T 细胞产生的方法，包括向有其需要的哺乳动物施用有效量的抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物，其中所述 T 细胞识别恶性细胞上的抗原。

[0091] 在某些实施方案中，所述化合物不是 Val-boroPro。

[0092] 在某些其他实施方案中，所述化合物诱导选自 GCSF 和 CXCL1 的细胞因子的产生。

[0093] 在某些其他实施方案中，所述 T 细胞产生得到刺激。

[0094] 在又其他实施方案中，所述 T 细胞产生得到增强。

[0095] 在仍又其他实施方案中，所述恶性细胞是癌、肉瘤、白血病、淋巴瘤或骨髓瘤。

[0096] 在某些实施方案中，所述哺乳动物是灵长类动物、犬、马、猫或牛。

[0097] 在某些其他实施方案中，所述哺乳动物是人。

[0098] 在某些实施方案中，口服或肠胃外施用所述化合物。

[0099] 在某些其他实施方案中，肠胃外施用所述化合物。

[0100] 在又其他实施方案中，口服施用所述化合物。

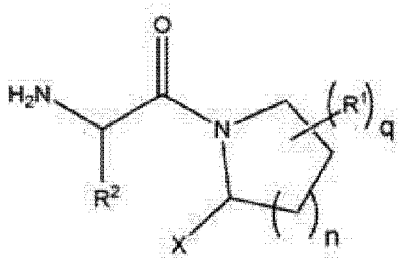
[0101] 在某些实施方案中，以固体剂型施用所述化合物。

[0102] 在某些其他实施方案中，所述固体剂型是片剂、胶囊或丸剂。

[0103] 在又其他实施方案中，所述固体剂型是片剂。

[0104] 在某些实施方案中，以足以刺激免疫系统而没有剂量限制性毒性的量施用所述化合物。

[0105] 在某些实施方案中，本发明涉及上述方法中的任一种，其中所述化合物由式 I 代表：



式 I

其中：

X 是 $B(Y^1)(Y^2)$ 或 CN；

Y^1 和 Y^2 独立地为 OH，或与它们连接的硼原子一起代表可水解为硼酸的基团，或与它们连接的硼原子一起形成可水解为硼酸的 5 至 8 元环；

R^1 选自卤素、低级烷基、低级烯基、低级炔基、羰基、羧基、酯、甲酸酯、酮、硫代羰基、硫酯、硫代乙酸酯、硫代甲酸酯、氨基、酰基氨基、酰胺基、硝基、硫酸酯、磺酸酯、磺酰胺基、$-(CH_2)_m-R_7$、$-(CH_2)_m-OH$、$-(CH_2)_m-O-$ 低级烷基、$-(CH_2)_m-O-$ 低级烯基、$-(CH_2)_n-O-(CH_2)_m-R_7$、$-(CH_2)_m-SH$、$-(CH_2)_m-S-$ 低级烷基、$-(CH_2)_m-S-$ 低级烯基、或 $-(CH_2)_n-S-(CH_2)_m-R_7$、叠氮基、氰基、异氰酸根合、硫氰酸根合、异硫氰酸根合、氰氧基、  、或  ；

R_7 代表取代或未取代的芳基、芳烷基、环烷基、环烯基或杂环；

R_8 独立地代表氢、$-CH_3$ 或 $-(CH_2)_n-CH_3$；

M 是 0、1、2、3、4、5 或 6；

R^2 是选自正丙基、C_4-C_8 烷基、C_2-C_8 烯基、C_2-C_8 炔基、C_3-C_8 环烷基、C_2-C_7 杂环基、芳基、杂芳基、芳烷基、杂芳烷基、和天然存在的疏水性氨基酸的侧链的疏水基团；

n 是 0、1 或 2；且

q 是 0、1、2、3 或 4。

[0106] 在某些实施方案中，q 是 0、1 或 2。

[0107] 在某些其他实施方案中，q 是 0。

[0108] 在又其他实施方案中，n 是 0。

[0109] 在仍又其他实施方案中，n 是 1。

[0110] 在某些其他实施方案中，n 是 2。

[0111] 在某些实施方案中，X 是 $B(Y^1)(Y^2)$。

[0112] 在某些其他实施方案中，X 是 $B(OH)_2$。

[0113] 在某些实施方案中，n 是 1；q 是 0；且 X 是 $B(OH)_2$。

[0114] 在某些实施方案中，R^2 选自叔丁基、异丁基、戊基、环己基、苄基或萘基。

[0115] 在某些其他实施方案中，R^2 选自叔丁基、异丁基或戊基。

[0116] 在仍又其他实施方案中，R^2 是叔丁基。

[0117] 在某些实施方案中，R^2 是天然存在的疏水性氨基酸的侧链。

[0118] 在某些其他实施方案中，R^2 是亮氨酸、异亮氨酸、叔亮氨酸、苯丙氨酸或色氨酸的侧链。

[0119] 在某些实施方案中，式 I 的化合物是叔丁基 Gly-boroPro。

[0120] 在某些实施方案中，在携带 X 的碳处的立体化学构型是 L。

[0121] 在某些其他实施方案中，在携带 X 的碳处的立体化学构型是 D。

[0122] 在某些实施方案中，在携带 R^2 的碳处的立体化学构型是 L。

[0123] 在某些其他实施方案中，在携带 R^2 的碳处的立体化学构型是 D。

[0124] 在某些实施方案中，在携带 X 的碳处的立体化学构型是 L；且在携带 R^2 的碳处的立

体化学构型是L。

[0125] 在某些其他实施方案中，在携带X的碳处的立体化学构型是L；且在携带R^2的碳处的立体化学构型是D。

[0126] 在又其他实施方案中，在携带X的碳处的立体化学构型是D；且在携带R^2的碳处的立体化学构型是L。

[0127] 在某些实施方案中，在携带X的碳处的立体化学构型是D；且在携带R^2的碳处的立体化学构型是D。

[0128] 术语“DASH丝氨酸蛋白酶”是指二肽基肽酶(DPP)IV活性和/或其结构同源物。这些蛋白是通过它们共同的脯氨酸后裂解(post-proline-cleaving)的丝氨酸二肽酶机制联合的酶。例如，DPP-VII，原名为静止期细胞脯氨酸二肽酶(QPP)，是DASH丝氨酸蛋白酶。

[0129] Val-boroPro，也被称为PT-100或talabostat，似乎经由巨噬细胞中胱天蛋白酶-1的活化和IL-1β的诱导来刺激免疫力，其进而上调巨噬细胞和基质成纤维细胞中细胞因子和趋化因子表达。细胞内DPP 8和/或9活性似乎是巨噬细胞中PT-100的相关目标。这种作用机理表明免疫系统中的细胞内DPP的迄今无法预料的调节作用。

[0130] ARI-4175，叔丁基(tertiary-butyl)(简称叔丁基(*t*-butyl))Gly-boroPro，是一种二肽硼酸，其作为用于治疗癌症的树突状细胞疫苗的佐剂有效地抑制丝氨酸蛋白酶的脯氨酰肽酶家族的所有六个成员。与PT-100类似，ARI-4175抑制DPP8/9活性。预期其他二肽硼酸，优选具有庞大疏水侧链的二肽硼酸，诸如异亮氨酸-boroPro、丁基甘氨酸-boroPro、苯丙氨酸-boroPro(Phe-boroPro)和环己基甘氨酸-boroPro(Cyg-boroPro)以类似的方式发挥作用。本领域技术人员进行常规实验可以确定所要求保护的方法中可以成功地使用何种抑制多种哺乳动物DASH丝氨酸蛋白酶的化合物(例如，式I的化合物)。

[0131] PT-100经由肿瘤和引流淋巴结中细胞因子/趋化因子上调来活化小鼠中的肿瘤免疫力。细胞因子作为癌症疫苗佐剂的用途不是新的：例如，用于sipuleucel-T的GM-CSF，和用于开发中疫苗的GM-CSF或IL-2和IFN-γ。然而，与这些应用相比，口服活性DPP8/9抑制剂，PT-100和ARI-4175，具有刺激肿瘤相关巨噬细胞和基质细胞以产生细胞因子和趋化因子的组合的优点，所述细胞因子和趋化因子的组合可以合作来活化肿瘤特异性效应T细胞。在由PT-100上调的细胞因子和趋化因子中，IL-1β、CXCL9和CXCL10是特别值得注意。肿瘤相关巨噬细胞产生的IL-1β在活化促炎应答和促进肿瘤微环境中T_h17细胞的发育中发挥关键作用。

[0132] 基于强大的临床前抗肿瘤活性和新型作用机制，PT-100进展到癌症的人体试验，并且由FDA授予快速追踪标识(fast-track designation)。然而，尽管在非霍奇金氏淋巴瘤(NHL)、转移性黑色素瘤和非小细胞肺癌(NSCLC)的非随机II期研究的临床活性中存在一些信号，但PT-100最终没能满足其在NSCLC的关键III期试验中的目标。两个因素可能促成这种失败。最重要地，临床前研究表明，对于PT-100在小鼠中的最佳抗肿瘤活性，对肿瘤的内源性免疫应答是需要的。不可能任何此类潜在肿瘤免疫力保留在III期中研究的晚期NSCLC患者中。其次，癌症患者中的剂量限制性毒性看起来阻止将对于一致免疫刺激患者足够高的PT-100剂量施用于患者。Fry等人的研究表明，当用来加强癌症疫苗时，PT-100的作用机制应该是临床上最有效的。尽管当与可以引发肿瘤特异性T细胞的适当疫苗使用

时，可能 PT-100 可以是临床上成功的；但本发明的目标是鉴定将在人中获得临床成功的具有较低毒性的类似物。

[0133] 单独或与树突状细胞治疗 (DCT)、曲妥珠单抗、西妥昔单抗、易普利姆玛、维罗非尼、索拉非尼或其他癌症免疫治疗组合的 ARI-4175 或抑制多种哺乳动物 DASH 丝氨酸蛋白酶的其他化合物（例如，式 I 的化合物）相对于其他癌症免疫治疗具有显著优势，因为它们是口服活性小分子。它们引发与困难且昂贵的 DCT 或抗体治疗类似的免疫活化，但施用可以更容易得多。ARI-4175 和抑制多种哺乳动物 DASH 丝氨酸蛋白酶的化合物（例如，式 I 化合物）是第一个它们种类的“口服活性肿瘤抗原”。

[0134] 许多患者对西妥昔单抗没有应答，或对治疗初步应答后发展出耐受性。这是由于癌症发展出对患者的免疫系统的耐受性。免疫应答仍然存在，但不再强大到足以杀死肿瘤，或者肿瘤变得对免疫系统是不可见的。一个实例是在约 40％的恶性结肠直肠癌中发现的 KRAS 突变。一项新近临床试验 (Lièvre 等人，*Cancer Res.* 2006, 66 (8), 3992) 发现，KRAS 突变与对西妥昔单抗的耐受性相关，而所有对西妥昔单抗应答的患者都缺乏 KRAS 突变。尽管西妥昔单抗产生临床应答的分子机制仍然未知，但用单独或与西妥昔单抗或其他免疫治疗组合的 ARI-4175 重新活化免疫应答可以改善其免疫应答不足以杀死肿瘤或具有难治性癌症的患者中的临床结果。

[0135] ARI-4175 或抑制多种哺乳动物 DASH 丝氨酸蛋白酶的其他化合物（例如，式 I 化合物）也可以与 T 细胞过继性转移治疗组合使用。这种治疗方法使用基于 T 细胞的细胞毒性来攻击癌细胞。ARI-4175 的佐剂性质允许其用作施用肿瘤浸润淋巴细胞或 TIL 之前的预处理，或用作施用过继性细胞转移之后的后处理。

[0136] ARI-4175 或抑制多种哺乳动物 DASH 丝氨酸蛋白酶的其他化合物（例如，式 I 化合物）的低毒性允许其用作癌症目前在缓解中的癌症患者中的佐剂。此类患者将受益于增加的抗肿瘤免疫应答以避免复发。

[0137] 预期 ARI-4175 和抑制多种哺乳动物 DASH 丝氨酸蛋白酶的其他化合物（例如，式 I 化合物）可以与 CTLA4 抑制剂诸如易普利姆玛 (Yervoy®)（这是一种增加免疫活性的受体拮抗剂）协同发挥作用。CTLA4（细胞毒性 T 淋巴细胞抗原 4），也称为 CD152，是一种下调免疫系统的蛋白受体。与 CD28 受体激动剂组合的 ARI-4175 或抑制多种哺乳动物 DASH 丝氨酸蛋白酶的其他化合物（例如，式 I 化合物）将以类似方式起作用以增加 T 细胞活性。

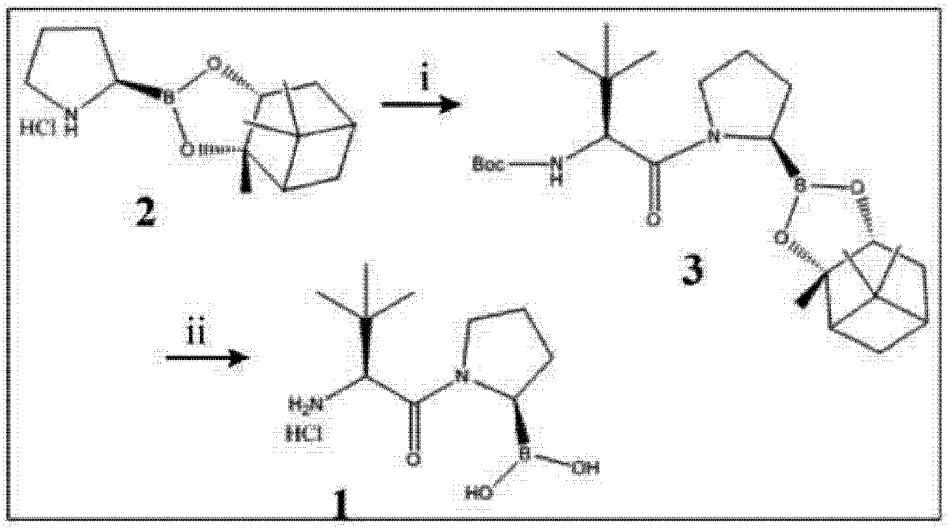
实施例

[0138] 本发明现在进行总体描述，其通过参考以下实施例将更容易理解，包括所述实施例仅仅是为了举例说明本发明的某些方面和实施方案的目的，并且不旨在限制本发明。

[0139] *实施例 1*

基本原理、化合物的合成和 RMS 肿瘤生长的抑制

ARI-4175 的合成。使用 HATU 将商售的 L-boroPro-pn 2 偶联到 *N*-Boc 保护的非天然氨基酸 Boc-Tle-OH 3 (CAS 号 62965-35-9)，以产生受保护的二肽硼酸酯 Boc-Tle-boroPro-pn。通过三氯硼烷 (BCl_3) 同时去除两个保护基团，随后通过反相 HPLC 纯化得到作为 HCl 盐的所需产物 1 (ARI-4175)。



方案 1. ARI-4175 的合成方案。试剂和条件：(i) Boc-L-Tle-OH, HATU/DIPEA/DMF, (ii) BCl_3。

[0140] PT-100 的合成。如前所述以足够用于以下实施例中所述的研究的量合成 PT-100。

[0141] ARI-4175 是 DPP-IV- 样丝氨酸蛋白酶（包括 DPP 8 和 9（表 1））的纳摩尔抑制剂，所述 DPP 8 和 9 是 PT-100 的免疫作用机制的假定目标。DPP-IV 和 FAP 活性的抑制也可以对 PT-100 的抗肿瘤效果有贡献，因为 DPP-IV 或 FAP 活性的选择性废除似乎减缓肿瘤的生长。

表1. DPP-IV-样蛋白酶活性在体外的抑制效力

抑制剂	IC_{50}[1] (nM)					
	DPP-IV	DPP8	DPP9	DPP-II	FAP	PREP[2]
PT-100 (Val-boroPro)	0.7	3.6	1.7	8.2	17.0	35.0
ARI-4175 (叔丁基Gly-boroPro)	1.6	5.1	1.9	88.0	32.0	24.0

[1] 50%平均抑制浓度

[2] 脯氨酰内肽酶

[0142] 在肿瘤接种后第 10 天用 RMS DC 疫苗在后肢肌内接种具有已建立的 RMS 的 C57BL/6 小鼠。如本文所述从第 10 天起持续 3 个周期（每次 5 天）通过每日管饲法施用 ARI-4175、PT-100 或媒介物 ：ARI-4175, 10 mg/kg, 第 1 个周期和 5 mg/kg 第 2 和 3 个周期 ；PT-100, 1 mg/kg 第 1-3 个周期。另一组小鼠接受疫苗但是未接受化合物，未接种小鼠组接受化合物或媒介物。每个方案中处理 8 只重复小鼠。

[0143] 如图 8 中显示，施用 ARI-4175 自身显著减缓肿瘤生长（图 8A）并且到第 25 天在 3/8 小鼠中产生肿瘤消退（图 9）。与疫苗组合，ARI-4175 在 6/8 小鼠中产生消退（图 9），并且显著增加小鼠存活率 ($P = 0.0045$ ；图 1B）。与此相反，以 1 mg/kg 的剂量施用的 PT-100，无论有或没有接种 DC，都未能到第 25 天产生肿瘤消退（图 2）或肿瘤生长的显著抑制（图 8A），并且用 PT-100 和疫苗处理的 6 只小鼠中仅 2 只中肿瘤生长降低（图 9）。先前显示

PT-100 的 1 mg/kg 剂量对于 C57BL/6 小鼠中肿瘤免疫的活化是最优的，且剂量不能增加高得多，因为在C57BL/6小鼠中PT-100的MTD为~2 mg/kg。因此，10/5-mg/kg剂量的ARI-4175的显著疫苗佐剂效应表明，ARI-4175 比 PT-100 的毒性更低，而且，在 RMS 小鼠模型中，与以耐受剂量的 PT-100 的可能性相比，可能增加 ARI-4175 的剂量来实现更大的肿瘤消退和小鼠存活。

[0144] *实施例 2*

ARI-4175 在 RMS DC 肿瘤疫苗模型中的有效性

图 17 A 显示了用于引发 T 细胞供体和 T 细胞受体的实验设置。图 17 B 显示肿瘤体积曲线（平均值 ± 标准偏差）和存活曲线。与盐水相比，单独接受ARI-4175的小鼠具有显著较小的肿瘤 (n=10, p=0.0019)。尽管 ARI-4175 + 引发的 T 细胞受体具有较小的肿瘤，但当与 ARI-4175 + 未引发的 T 细胞受体相比时，差异不显著 (n=10, p=0.0755)。十个 ARI-4175 + 引发的 T 细胞受体中的八个存活到第 80 天，然而，这与 ARI-4175 + 未引发的 T 细胞受体的 40% 存活率相比不显著 (n=10, p=0.0658)。

[0145] 图 18 A 和 B 显示，Rag1-/- 受体中用 ARI-4175 和过继性 T 细胞转移联合治疗显著降低 RMS M3-9-M 体积。(A) 雌性 $Rag1^{-/-}$ 小鼠在肿瘤攻击后一天接受未引发的或 RMS 引发的 T 细胞。(B) 到第 10 天，ARI-4175 治疗的小鼠与盐水治疗的小鼠相比具有显著较小的肿瘤（未引发的 :n=5, p=0.0159 ;引发的 :n=5, p=0.0079）。肿瘤引发的 T 细胞与 ARI-4175 组合治疗的 $Rag1^{-/-}$ 小鼠具有总体最小的肿瘤。

[0146] ARI-4175 是 DC 接种的有效的佐剂，如显著改善的存活和降低的 RMS M3-9-M 体积（图 19）所证明。图 19 A 显示了 DC 接种和 ARI-4175 治疗的晚期治疗模式。图 19 B 显示肿瘤体积曲线（平均值 ± s.d.）和存活曲线。与对照相比，用 ARI-4175 治疗的两组都具有明显改善的存活率（假疫苗 :n=7, p<0.001，DC 疫苗 :n=7, p<0.001）。与用单独的 ARI-4175 治疗的小鼠相比，使用 ARI-4175 和 DC 疫苗的联合治疗治疗的小鼠具有显著较小的肿瘤 (n=7, p=0.0481)。

[0147] RMS 细胞系衍生自肝细胞生长因子 / 分散因子 (HGF/SF) 转基因的 *Ink4a/Arf*$^{-/-}$ 小鼠，其发展具有高外显率恶性 RMS。DC 疫苗将通过如前所述将骨髓衍生的 DC 与从 RMS 细胞生成的凋亡小体孵育来制备。肿瘤生长每 2 天通过卡尺测量监测。

[0148] *实施例 3*

由 Ala-boroPro (2243)、Val-boroPro (PT-100, 2054) 和 *t*-BuGly-boroPro (ARI-4175) 诱导 IL-1β 并上调肿瘤和引流淋巴结中的细胞因子和趋化因子表达

BALB/c 小鼠中细胞因子测定方法。通过口管饲法 (PO) 或腹膜内 (IP) 注射用各种剂量的 PT-100 或 ARI-4175 治疗雌性 BALB/c 小鼠并分析血清的趋化因子（图 4、5 和 6）。在给药后各个时间通过心脏穿刺收集血液，并制备血清用于通过 ELISA 分析。使用来自 R&D Systems 的 ELISA 试剂盒（目录号分别为 MCS00 和 MKC00B）测定样品的小鼠小鼠细胞因子 G-CSF 和小鼠 CXCL1。一式两份进行所有测量。必要时稀释血清样品以获得测定的范围内的值。所需最佳稀释度根据测试试剂而不同，范围可以从没有稀释（对于对照样品或没有活性的测试试剂）到 1:1000 稀释（对于非常高的样品）。对于产生阳性应答的试剂，对于 CXCL1 在给药后 2 小时且对于 G-CSF 在给药后 6 小时观察到最强信号。剂量应答随着检测试剂而不同，但是 20 μg/ 小鼠剂量对于应答评估是可接受的基线剂量。通常，在每个时间点

对于每种试剂测量 6 只动物。

[0149] 图 4 显示，Val-boroPro (2054)，但不是 Ala-boroPro (2243) 刺激 BALB/c 小鼠中的 G-CSF 和 CXCL1/KC。G-CSF 和 CXL1/KC 是抗癌免疫增强活性的标记物。

[0150] ARI-4175（图 5 和图 6 中的 4175-2）是细胞因子的一种非常有效的诱导物。G-CSF 的刺激增加直至施用后 6 小时；CXCL1 在施用后 3 h 被迅速诱导，但到 6 h 时已经消失（图 5）。ARI-4175 (4175-2) 在诱导 G-CSF 和 CXCL1 细胞因子时比 PT-100 (2054) 效力高至少 5 倍（图 6）。来自 ARI-4175 治疗小鼠的血清与媒介物相比也已经增加了 IL-18、IL-1β 和 IFN-γ，但水平比 G-CSF 和 CXCL1 低得多。

[0151] 口服施用至携带肿瘤的小鼠后 2 小时，PT-100 刺激促炎性细胞因子和趋化因子 mRNA 的表达。PT-100 应答的特征在于肿瘤和淋巴结组织中 IL-1β、G-CSF、IL-6、CXCL1、CXCL9 和 CXCL10 的上调。最近发现 ARI-4175 在体外刺激产生 IL-17 的 T_h17 细胞的发育 (V. Kuchroo，未发表的数据）。T_h-17 细胞似乎对某些癌症中的有效抗肿瘤免疫力有贡献；因此，IL-17 将包括在细胞因子 / 趋化因子实验对象组，所述细胞因子 / 趋化因子实验对象组用来表征在 ARI-4175 和 PT-100 施用于 RMS-DC 接种小鼠后我们将研究的对 PT-100 的应答。以最佳剂量施用化合物后 2 小时，将测定 RMS 肿瘤和引流淋巴结组织中的 RNA 表达。我们将使用先前使用的 RT-PCR 程序来分析携带 A549 肺癌异种移植物的小鼠中 PT-100 对细胞因子和趋化因子的上调。cDNA 将用 iScript 试剂盒 (Biorad, Hercules, CA) 从通过 Trizol (Invitrogen, Carlsbad, CA) 提取的总 RNA 合成，在水中 1:10 稀释，并在 2X iQ Sybergreen Supermix (Biorad) 中使用 10-μM 未标记的引物对在热循环仪中扩增 40 个循环 (cDNA 变性，95℃ /15 s；退火和延伸，60℃ /30 s)。反应将使用用 HEX、FAM 或 Texas Red 5′ - 标记的和用黑洞猝灭剂 (Biosearch Technologies, Novato, CA) 3' - 标记的 Taqman 探针。将使用 Beacon Designer 软件 (Premier Biosoft International, Palo Alto, CA) 设计细胞因子 / 趋化因子目标 mRNA 和 18s RNA 参考对照正向 / 反向引物对和 TaqMan 探针。将用大小表征的参考 cDNA 使用标准曲线从循环阈值计算 mRNA 拷贝数，所述大小表征的参考 cDNA 将从小鼠组织 RNA 合成和扩增，通过电泳和凝胶纯化试剂盒 (Qiagen, Valencia, CA) 纯化，并通过 PicoGreen (Invitrogen, Carlsbad, CA) 定量。

[0152] 如图 11 和 12 中显示，用 ARI-4175 治疗小鼠也增加 NK 细胞表达 FcγRIII 受体、CD16 和脱粒标记物 LAMP-1。人 NK 细胞的体外治疗也增加活化标记物 CD69（图 15）。ARI-4175 的治疗效果可能部分是由于通过升高 CD16 (FcγRIIIA) 的表达和活化 NK 细胞（基于 CD69 上调）而增强 ADCC。

[0153] *实施例 4*

其中发生肿瘤消退和排斥的接种小鼠的肿瘤再攻击的免疫记忆

用于癌症的有效疫苗将具有建立免疫记忆的优点，所述免疫记忆在对初始治疗的临床应答之后可以保护免于散播性转移或肿瘤再生长。在原发性肿瘤排斥后至少 20-30 天通过肌肉内注射 10×10^6 RMS 细胞而再攻击实施例 1 中其中在 RMS DC 接种和随后 ARI-4175 或 PT-100 治疗后排斥肌内 RMS 肿瘤的小鼠。再持续 20-30 天监测小鼠的继发性肿瘤生长，而没有任何额外的治疗性处理。为了证明保护的免疫特异性，还用 C57BL/6 肿瘤细胞系，EL4 (ATCC, TIB-39) 攻击小鼠。测试 4 只小鼠组的免疫记忆。已经描述了表明 PT-100 治疗后肿瘤特异性记忆的类似实验。

[0154] 如图 7 中显示，在第 0 天用 $1x10^6$ RMS M3-9-M 肌内攻击雌性 C57BL/6 小鼠。在第 3-7、17-21、24-28、和 31-35 天用 200 μg ARI-4175 口服管饲小鼠。在第 56 天用 $5x10^5$ RMS M3-9-M 再攻击无肿瘤存活者，并在没有额外的 4175 治疗的情况下进行监测。再攻击之后，RMS M3-9-M 在所有小鼠中表现出初始生长和随后的排斥 (n=7, p=0.0175)。

[0155] *实施例 5*

测定 RMS DC 接种小鼠中肿瘤特异性 CTL

通过 ^{51}Cr 释放测定法先体外后体内测定接受实施例 1 中所述的 RMS DC 疫苗和 ARI-4175 或 PT-100 治疗的携带 RMS 肿瘤的小鼠的肿瘤引流淋巴结和脾脏中的 CTL。如前对于 EL4 肿瘤接种的 C57BL/6 小鼠中 PT-100 刺激的肿瘤特异性 CTL 应答的测量所述进行该测定。通过针对 RMS 相比于 EL4 细胞的细胞毒性的比较研究 CTL 的特异性。

[0156] *实施例 6*

C57BL/6 小鼠中 ARI-4175 和 PT-100 的 MTDs

先前，在以高于 MTD (~2 mg/kg/ 天) 的剂量施用 PT-100 的 C57BL/6 小鼠中，在死亡前没有观察到任何明显毒性体征；因此，在本实验中用于确定 MTD 的终点为死亡率。为了深入了解毒性的原因，研究病理组织学以确定血浆细胞因子和趋化因子水平的剂量应答。使用细胞因子 / 趋化因子测定法来确定毒性是否与 PT-100 的假定作用机制相关。初步实验表明，用 IL-1R 拮抗剂阿那白滞素阻断细胞因子 / 趋化因子应答不会改变 PT-100 在大鼠中的毒性，表明 PT-100 的毒性可能不是由于全身性细胞因子 / 趋化因子产生。

[0157] 大鼠毒性的概述。进行最大耐受剂量 (MTD) 研究来比较 Sprague Dawley 大鼠中 ARI-4175 (*t*-BuGly-boroPro) 和 PT-100 (Val-boroPro, ARI-2054) 的 MTD。通过皮下注射 (SQ) 或口服管饲法 (PO) 对动物给药。每个剂量使用六只动物。在给药后 30 分钟从尾部采集血液样品，所述血液样品用于测量血浆药物浓度。对于两种化合物的起始剂量为 0.05 mg/kg 体重，然后对于每种药物向上或向下调整剂量，以确定存在 100％存活的最大剂量。在 0.05 mg/kg 体重 SQ 的起始剂量，ARI-4175 和 PT-100 治疗都导致前 24 小时内至少一只动物死亡。0.01 mg/kg 体重剂量 (SQ) 没有导致任何死亡，并且给药后持续 48 小时没有观察到任何副作用。以 0.025 mg/kg 体重的剂量进行第三次实验，其在 ARI-4175 组中导致一例死亡，但在 PT-100 组中没有导致死亡。用口服给药重复研究，再次以 0.05 mg/kg 体重开始。两种药物显然更被口服给药耐受，因为在该实验中在 0.05 mg/kg PO 没有任何副作用。将剂量增加至 0.1 mg/kg PO 在 PT-100 组中导致 2 例死亡，但用 ARI-4175 没有任何副作用。在 0.25 mg/kg ARI-4175 PO，有一例死亡。因此，对于 SQ 观察到的 MTD 对于 PT-100 为 0.025 mg/kg，对于 ARI-4175 为 0.01 mg/kg。通过口服途径，MTD 对于 PT-100 为 0.05 mg/kg，对于 ARI-4175 为 0.1 mg/kg。血浆药物浓度的评估表明无论施用途径的在等效血浆药物浓度的毒性结果。对于导致 100 +/-50 nM 的血浆药物浓度的药物剂量观察到毒性。该数据总结在图 20 中。

[0158] 小鼠毒性血浆相比于剂量图。通过口服管饲法以高达 40 mg/kg 的 ARI-4175 和高达 10 mg/kg 的 PT-100 (ARI-2054) 的各种剂量每天治疗 C57BL/6 小鼠，持续 5 天。在第 5 天，在给药前和给药后 30 分钟采集血液，并通过 LCMS 测量血浆药物浓度。PT-100 的口服利用度比 ARI-4175 的口服利用度高 3-4 倍，如以 10 mg/kg 剂量的血浆浓度所证明。血浆浓度在测试的范围内与剂量近似成比例。在该实验中存活率为 100％，但所有组在 5 天治疗期内

都显示显著的体重减轻。结果显示在图 21 中。

[0159] 小鼠中 4175 的 PK。通过口服管饲法 (PO) 和通过腹膜内 (IP)、皮下 (SQ) 和静脉内 (IV) 注射在正常 BALB/c 小鼠中以药物的打开（线性）和闭合（环状）形式测量 ARI-4175 的药代动力学。通过将药物在 pH 2 在室温孵育过夜而制备药物的打开形式。 通过在 pH 7.4（在 PBS 中）孵育过夜而制备闭合形式。通过临施用前稀释到 PBS 中而中和打开（线性）样品。治疗组在下面列出（表 2）。

[0160] 表 2

组	剂量	途径	n
1	1mpk	PO	4
2	1mpk	IP	4
3	1mpk	SQ	4
4	0.5mpk	IV	6

[0161] 对于所有组从尾静脉收集血液，除了 IV 组。在尾静脉中进行 IV 注射，因此从远端部位（颌下静脉）收集血液。制备血浆样品并通过 LC-MS 测量各样品中的 ARI-4175 浓度。结果显示在图 22 中。

[0162] *实施例 7*

接受渐增剂量的 ARI-4175 和 PT-100 的小鼠中组织病理学的研究

将 3 只小鼠组用 RMS 细胞肌内接种并以一定剂量通过管饲法施用 PT-100 和 ARI-4175，所述剂量从实施例 1 中测定的 MED 增加至高达实施例 6 中测定的 MTD。肿瘤接种后第 10 天至第 14 天将给予各化合物的一次 5 天周期，在第 18 天，将肿瘤、引流淋巴结、脾、肝、肺和肾组织的样本在福尔马林中固定并在石蜡中包埋。将来自试验小鼠的 H & E 染色组织切片与来自对照小鼠的切片进行组织学比较。已经显示 PT-100 刺激实体瘤的白细胞浸润。肿瘤浸润的特征在于在肿瘤和基质组织的边界处聚集的中性粒细胞。来自 ARI-4175 治疗的小鼠的肿瘤切片与来自 PT-100 治疗的小鼠的切片的比较将决定 AR1-4175 是否也促进肿瘤浸润。可能 PT-100 的毒性是由非肿瘤组织的白细胞浸润引起的，所述白细胞浸润导致引起器官衰竭的炎症应答。因此，我们将检查用 PT-100 和 ARI-4175 治疗的小鼠中非肿瘤组织样品中的白细胞存在。

[0163] *实施例 8*

使用 IL-1 受体缺陷小鼠研究全身性细胞因子 / 趋化因子在毒性中的作用

对 PT-100 的细胞因子 / 趋化因子应答在 IL-1R1 缺陷的 B6.129S7-*Il1r1*^{tm1Imx}/J 小鼠 (Jackson Laboratory) 中消除；因此，如果毒性是由于全身性细胞因子 / 趋化因子的活性，则 IL-R1 突变小鼠中的 MTD 应当相对于同类系 C57BL/6 小鼠中显著增加。因此，血清 G-CSF 和 CXCL1 细胞因子的剂量应答将通过 ELISA (R&D Systems) 和 B6.129S7-*Il1r1*^{tm1Imx}/J 相比于 C57BL/6 对照小鼠中 ARI-4175 和 PT-100 的 MTD 进行比较。在以渐增剂量水平治疗 3 只小鼠组中测定 MTD。在化合物施用后 3 小时和 8 小时采样的血清中测定 G-CSF 和 CXCL-1 水平。如果 IL-1R1 缺陷小鼠对毒性耐受，并且如果实验 6 中的组织病理学揭示非肿瘤组织的白细胞浸润，则将 IL-1R1 缺陷和充足的小鼠进行组织学比较，以确定毒性是否与器官功能的炎症破坏相关。

[0164] *实施例 9*

ARI-4175 在 KRAS 突变的结肠直肠癌细胞系中潜在的抗肿瘤和免疫效应；共同施用 ARI-4175 与西妥昔单抗

西妥昔单抗 (CTX) 是许多恶性肿瘤中的有效治疗剂。目前的数据表明,约 40%的携带突变的 K-ras 的结肠直肠癌患者不会受益于该试剂。西妥昔单抗的抗肿瘤效果的可能机制是通过抗体依赖性细胞介导的细胞毒性 (ADCC) 介导的。本研究探讨 ARI-4175 作为单一药剂或与西妥昔单抗联合在治疗 K-ras 突变体结肠直肠癌异种移植物中活性的潜能。

[0165] 在体外和体内评估单独或与西妥昔单抗组合的 ARI-4175 的作用。在体外,K-ras 突变体结肠癌细胞系 DLD-1 和 HCT-116 的增殖在含有各种浓度的 ARI-4175 或西妥昔单抗的培养基中培养三天后检测 (图 10)。单独或与西妥昔单抗组合的 ARI-4175 (10 nM - 200 μM) 没有显示出对细胞培养中的 DLD-1 或 HCT-116 的显著细胞毒性 (图 10)。在体内,将携带 DLD-1 或 HCT-116 异种移植肿瘤的裸鼠随机分入四组,对照、单独 ARI-4175、单独西妥昔单抗和 ARI-4175 加上西妥昔单抗。将 ARI-4175 以 100 μg q.d 或 b.i.d 口服施用,并将西妥昔单抗以 200 μg 每周腹膜内注射。肿瘤测量每周进行两次。在小鼠中,ARI-4175 的应用以剂量依赖性方式显著阻断 DLD-1 和 HCT-116 肿瘤两者的生长 (图 13 A 和 B)。ARI-4175 与西妥昔单抗的组合导致了肿瘤大小的进一步下降,尽管没有统计学显著性,可能是由于较少的动物数量。单独的西妥昔单抗对 HCT-116 异种移植物没有显示任何治疗效果,但的确对 DLD-1 肿瘤具有适中的效力。

[0166] *实施例 10*

PT-100 和 ARI-4175 的药代动力学概况比较 ;这两种化合物之间的其他区别

如图 16 中显示,用 $1x10^6$ MB49 皮下攻击雌性 C57BL/6 小鼠。在第 3-7 和 10-14 天对小鼠进行口服管饲。 肿瘤体积通过卡尺测量监测。在 20 μg 剂量, PT-100 和 ARI-4175 两者都诱导抗肿瘤活性。以 200 μg 给药的 ARI-4175 在 5/5 只小鼠中诱导完全消退,而 PT-100 在相同剂量是毒性的。

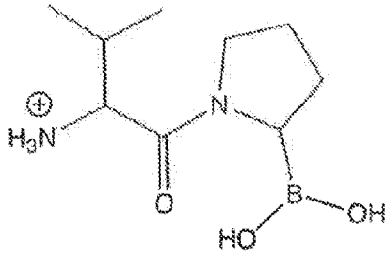
[0167] 尽管 PT-100 和 ARI-4175 之间的化学结构存在小差异,但两种化合物的药代动力学 (PK) 概况存在出乎意料的大差异。具体地,ARI-4175 的毒性低得多。

[0168] *通过引用并入*

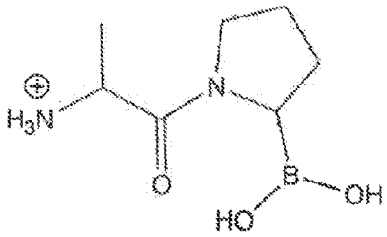
本文中引用的所有美国专利和美国专利申请出版物通过引用并入本文。

[0169] *等同方案*

仅仅使用常规实验,本领域技术人员将认识到或者能够确定本文所述的本发明的特定实施方案的许多等同方案。下列权利要求旨在涵盖此类等同方案。



Val-boroPro



Ala-boroPro

	K_i / IC50* (nM)							
	DPPIV	DPP8	DPP9	DPPII	FAP	PREP	MTD (mg/kg)	免疫刺激
V-bP	0.18	1.5	0.76	8.2*	17*	35*	0.05	G-CSF, IL-1β, INFγ
A-bP	0.027	2.0	0.53	1.4*	43*	240*	>200	无

体外 IC_{50} (nM)					
化合物	DPPIV	DPP8	DPP9	DPPII	FAP
PT-100	0.7	3.6	1.7	8.2	17
ARI-4175	1.6	5.1	1.9	88	32

图 1

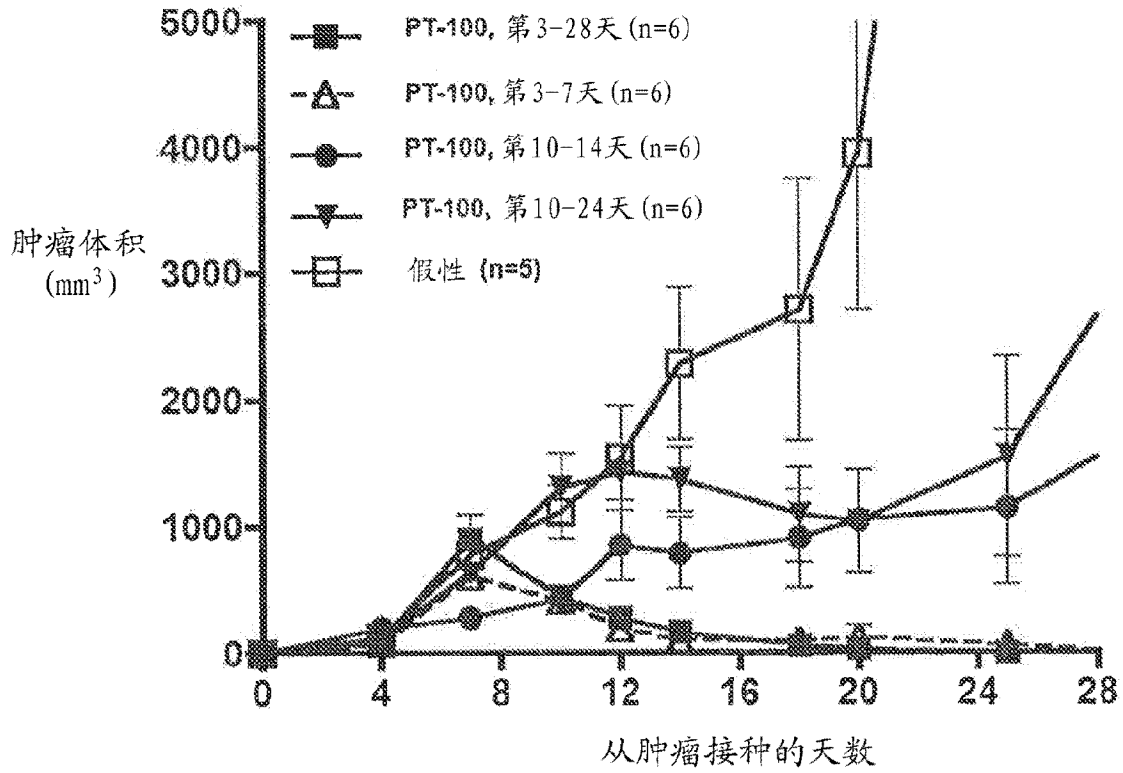

5000
4000
3000
2000
1000
0
肿瘤体积
(mm3)
PT-100, 第3-28天 (n=6)
PT-100, 第3-7天 (n=6)
PT-100, 第10-14天 (n=6)
PT-100, 第10-24天 (n=6)
假性 (n=5)
0
4
8
12
16
20
24
28
从肿瘤接种的天数

图 2

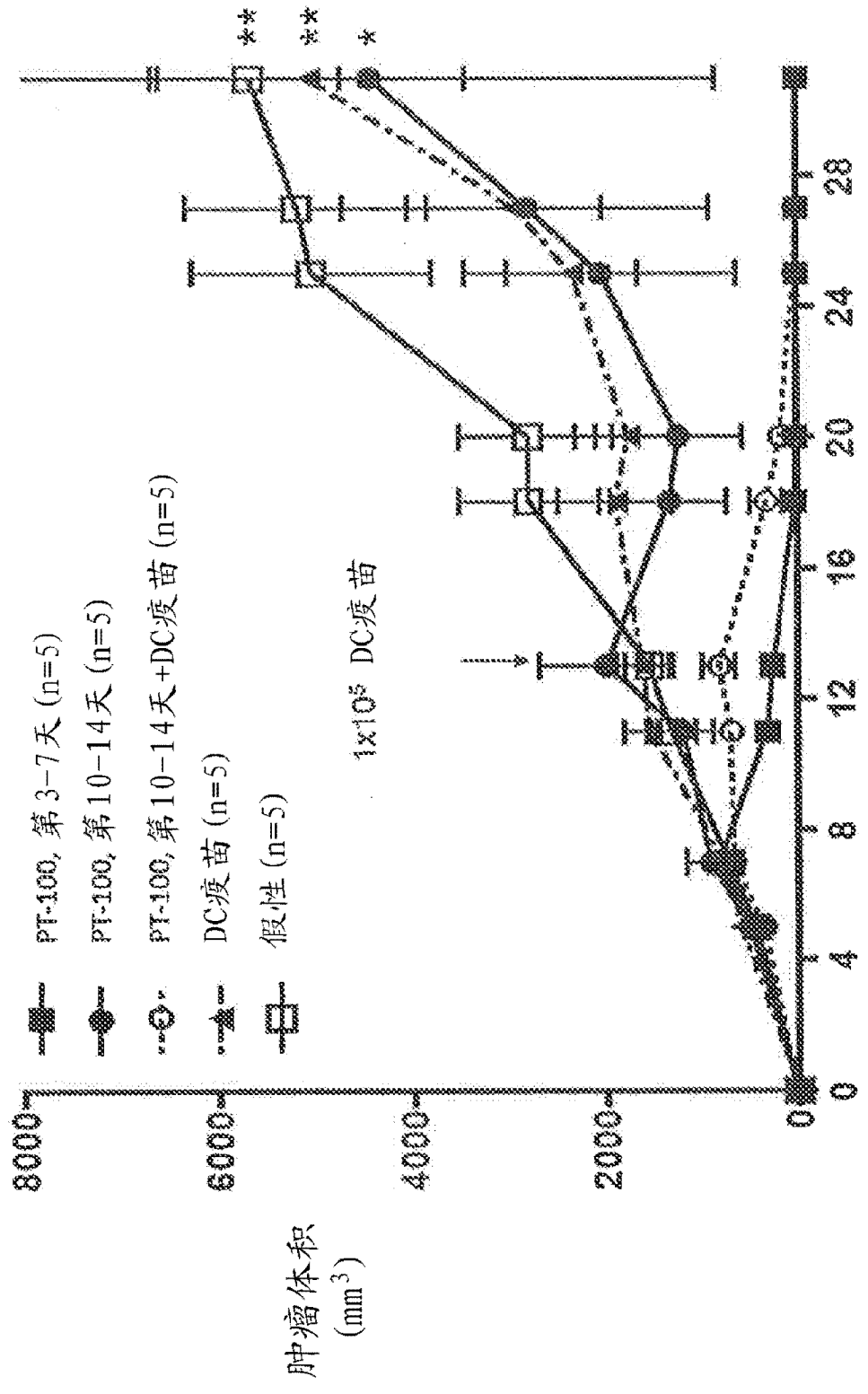

PT-100, 第3-7天 (n=5)
PT-100, 第10-14天 (n=5)
PT-100, 第10-14天+DC疫苗 (n=5)
DC疫苗 (n=5)
假性 (n=5)
1x10⁵ DC疫苗
肿瘤体积 (mm³)
8000
6000
4000
2000
0
0
4
8
12
16
20
24
28
**
**
*

图 3

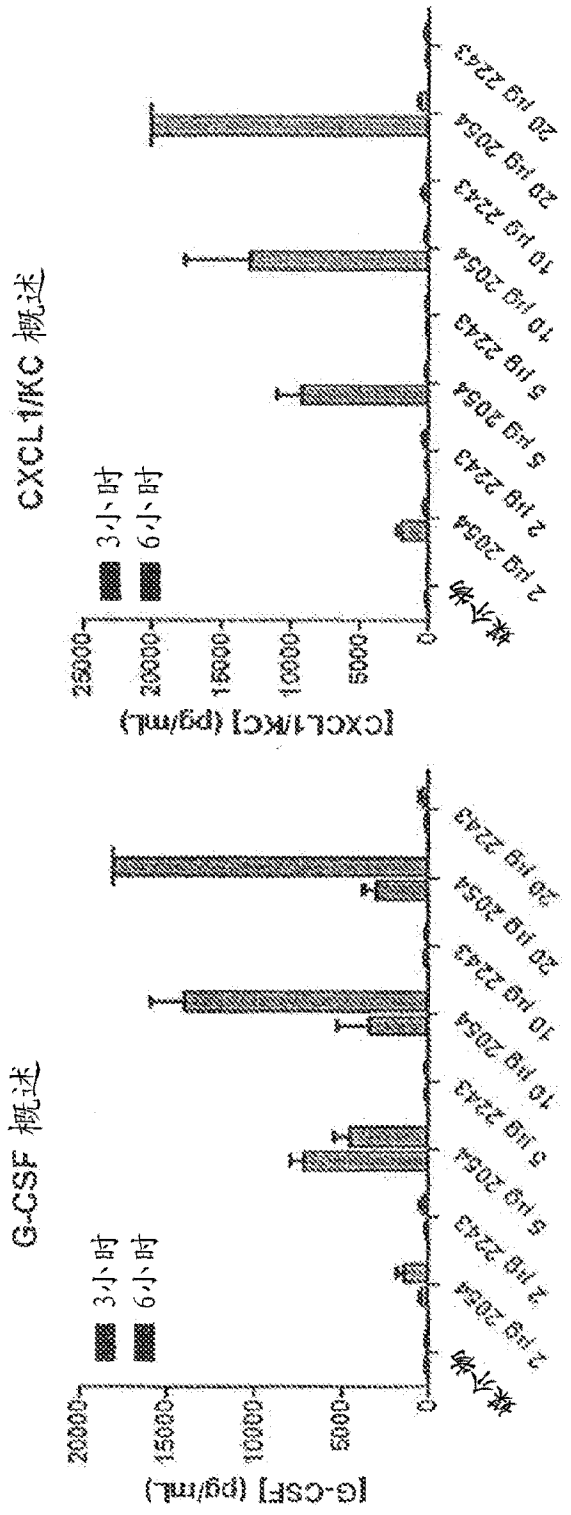

CXCL1/KC 概述
3小时
6小时
[CXCL1/KC] (pg/mL)
25000
20000
15000
10000
5000
0
媒介物
2 mg 2054
2 mg 2243
5 mg 2054
5 mg 2243
10 mg 2054
10 mg 2243
20 mg 2054
20 mg 2243
G-CSF 概述
3小时
6小时
[G-CSF] (pg/mL)
20000
15000
10000
5000
0
媒介物
2 mg 2054
2 mg 2243
5 mg 2054
5 mg 2243
10 mg 2054
10 mg 2243
20 mg 2054
20 mg 2243

图 4

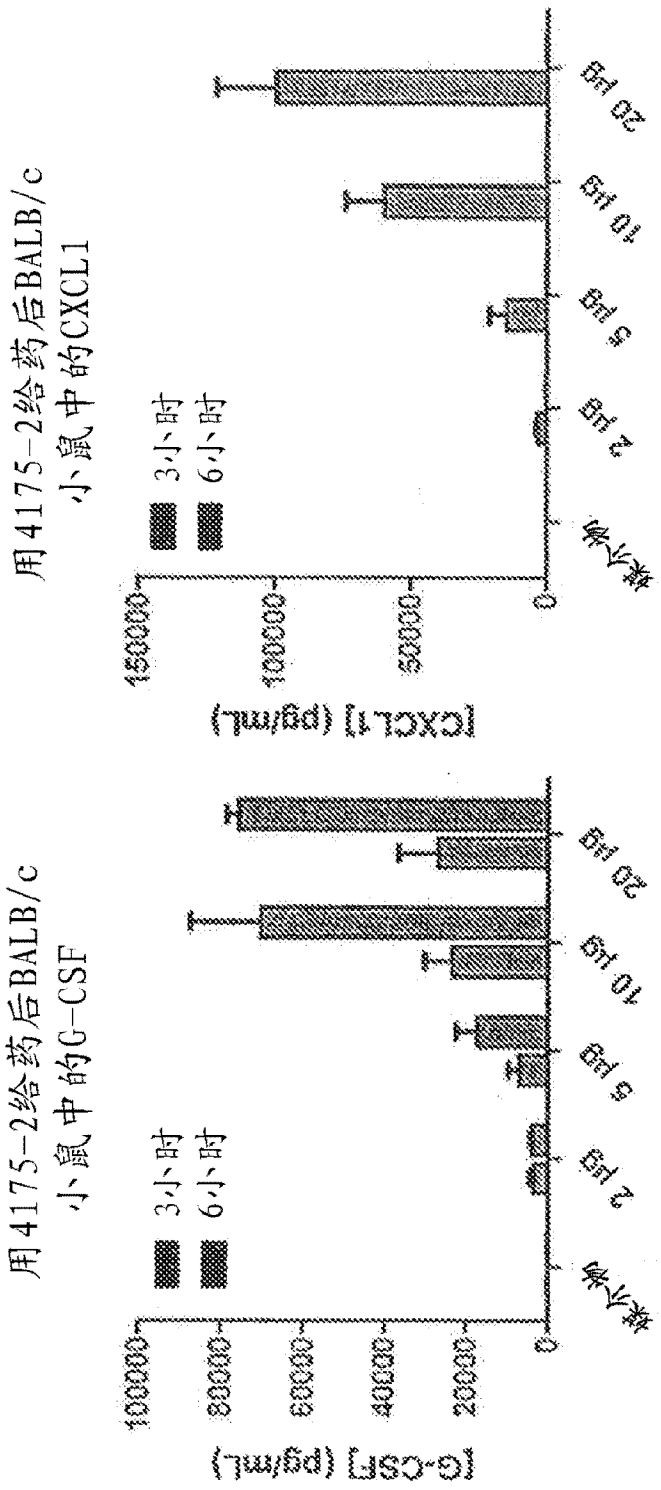

用4175-2给药后BALB/c
小鼠中的CXCL1
3小时
6小时
[CXCL1] (pg/mL)
150000
100000
50000
0
媒介物
2 mg
5 mg
10 mg
20 mg
用4175-2给药后BALB/c
小鼠中的G-CSF
3小时
6小时
[G-CSF] (pg/mL)
100000
80000
60000
40000
20000
0
媒介物
2 mg
5 mg
10 mg
20 mg

图 5

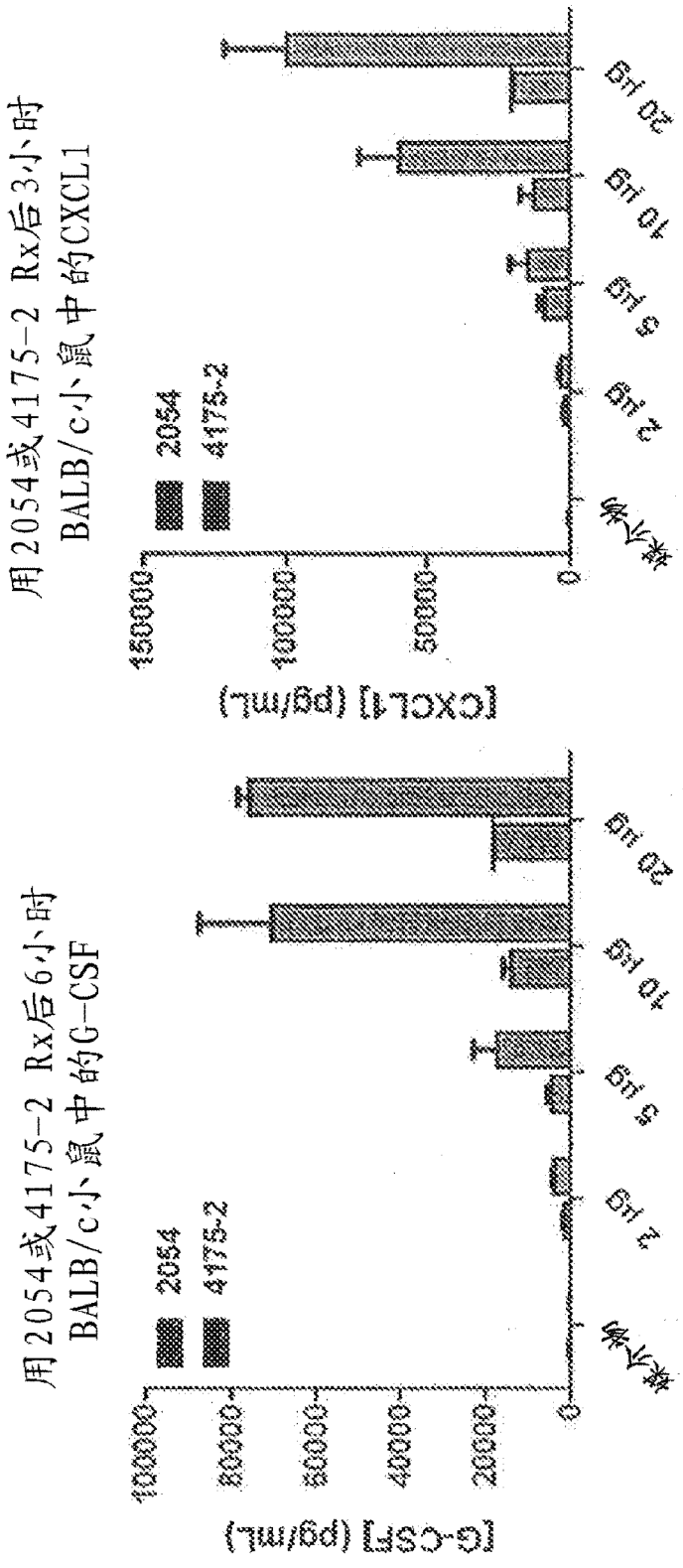

用2054或4175-2 Rx后3小时
BALB/c小鼠中的CXCL1
2054
4175-2
[CXCL1] (pg/mL)
150000
100000
50000
0
媒介物
Bri 2
Bri 5
Bri 10
Bri 20
用2054或4175-2 Rx后6小时
BALB/c小鼠中的G-CSF
2054
4175-2
[G-CSF] (pg/mL)
100000
80000
60000
40000
20000
0
媒介物
Bri 2
Bri 5
Bri 10
Bri 20

图 6

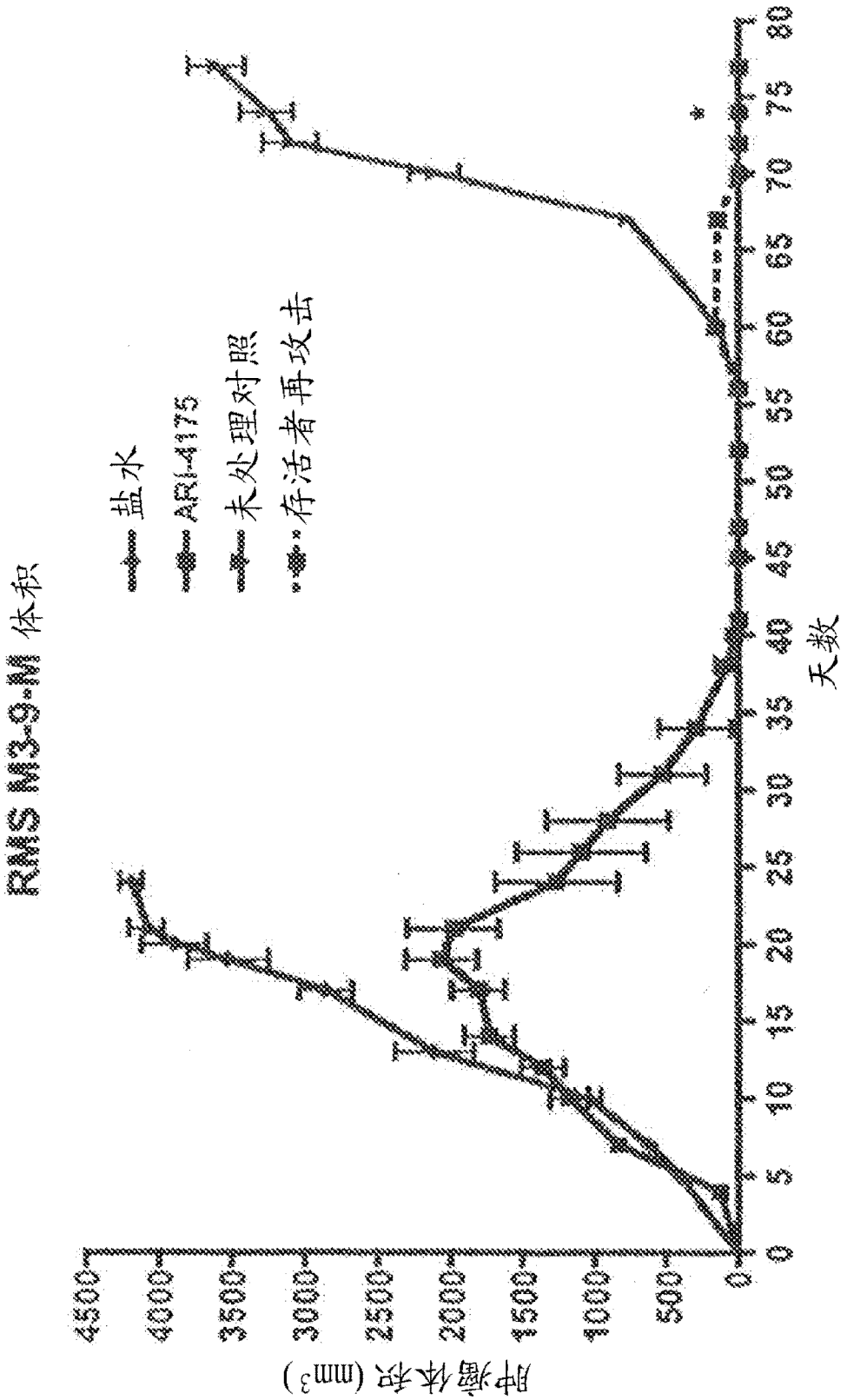

RMS M3-9-M 体积
盐水
AR1-4175
未处理对照
"存活者再攻击
肿瘤体积 (mm3)
天数
4500
4000
3500
3000
2500
2000
1500
1000
500
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80

图 7

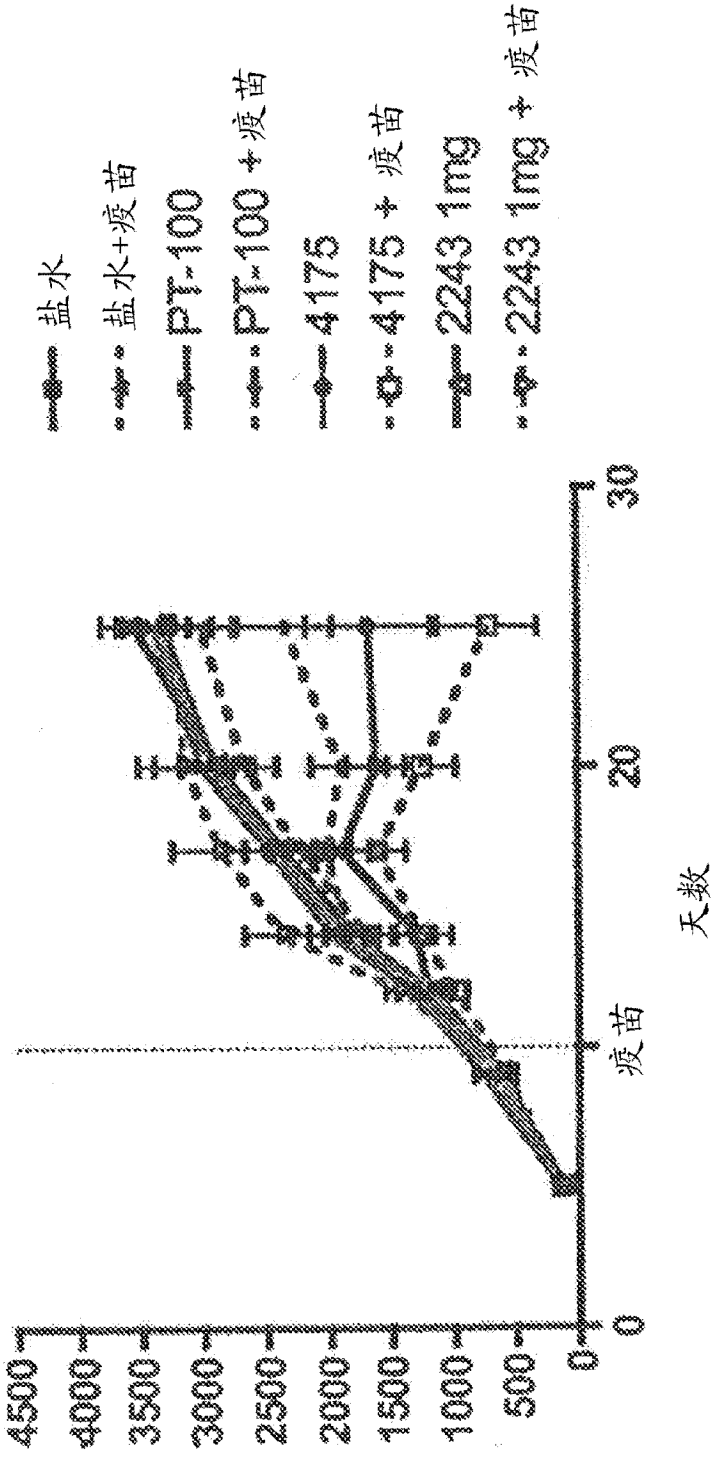

盐水
盐水+疫苗
PT-100
PT-100 + 疫苗
4175
4175 + 疫苗
2243 1mg
2243 1mg + 疫苗
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
30
疫苗
天数

图 8A

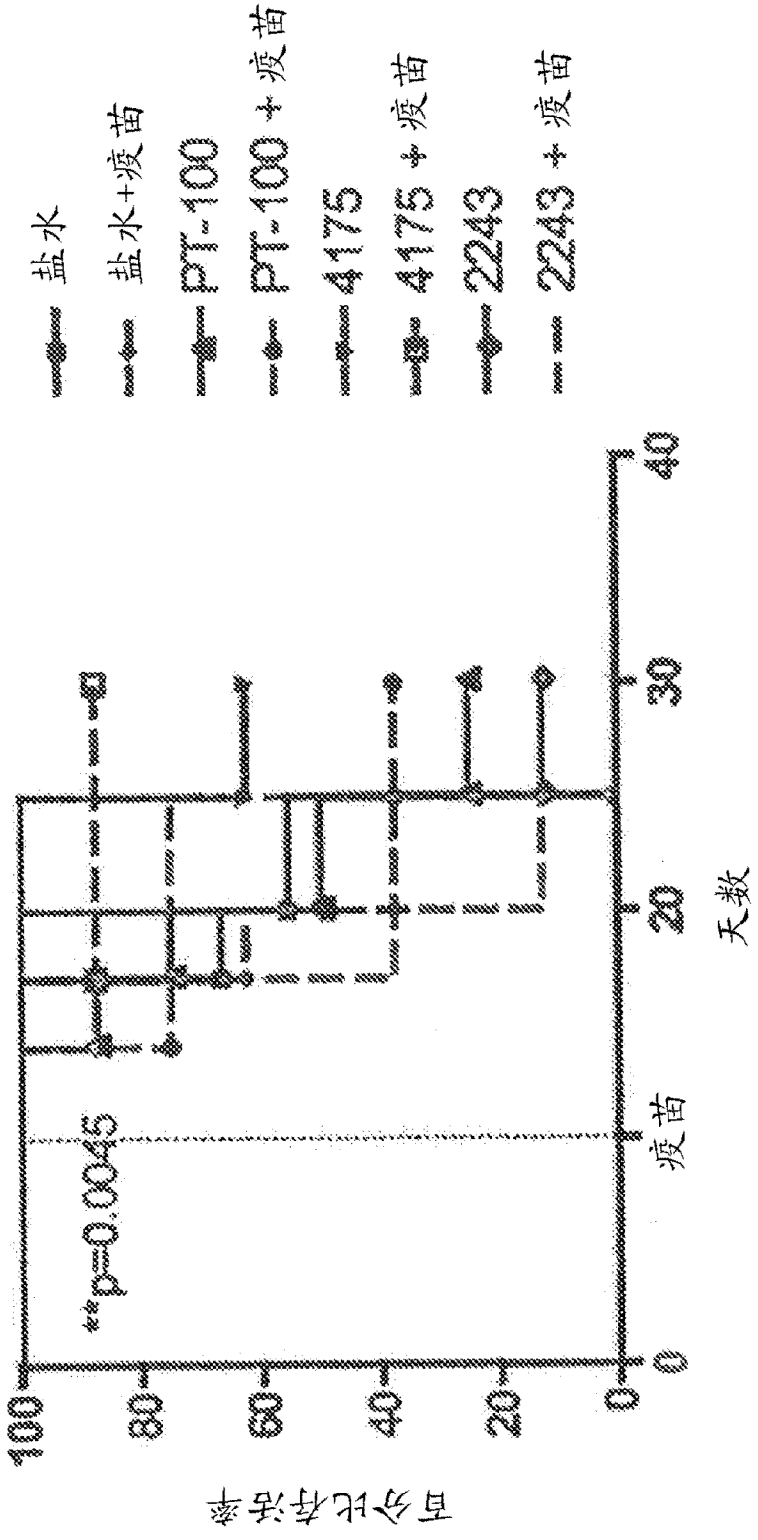

盐水
盐水+疫苗
PT-100
PT-100 + 疫苗
4175
4175 + 疫苗
2243
2243 + 疫苗
**p=0.0045
疫苗
天数
百分比存活率
100
80
60
40
20
0
0
20
30
40

图 8B

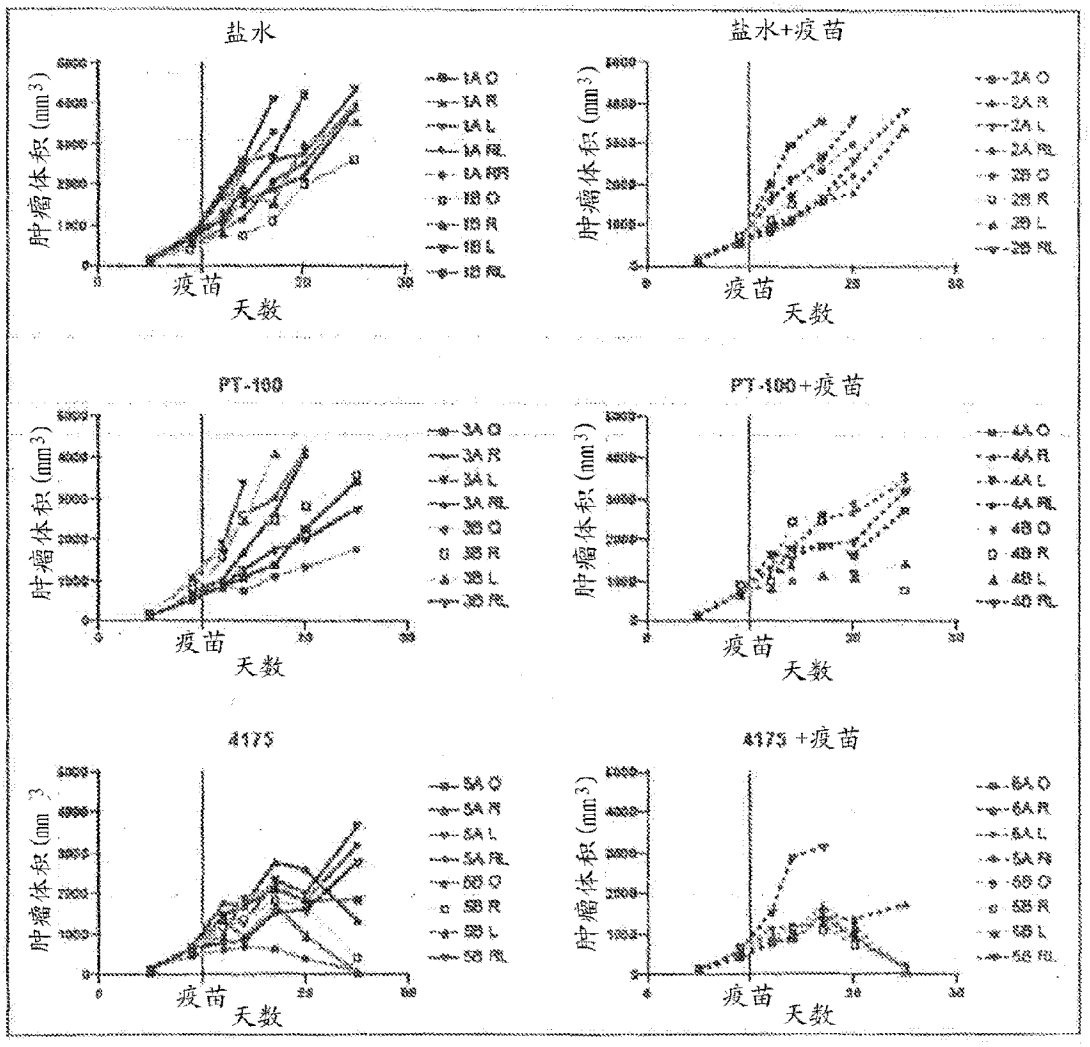


图 9

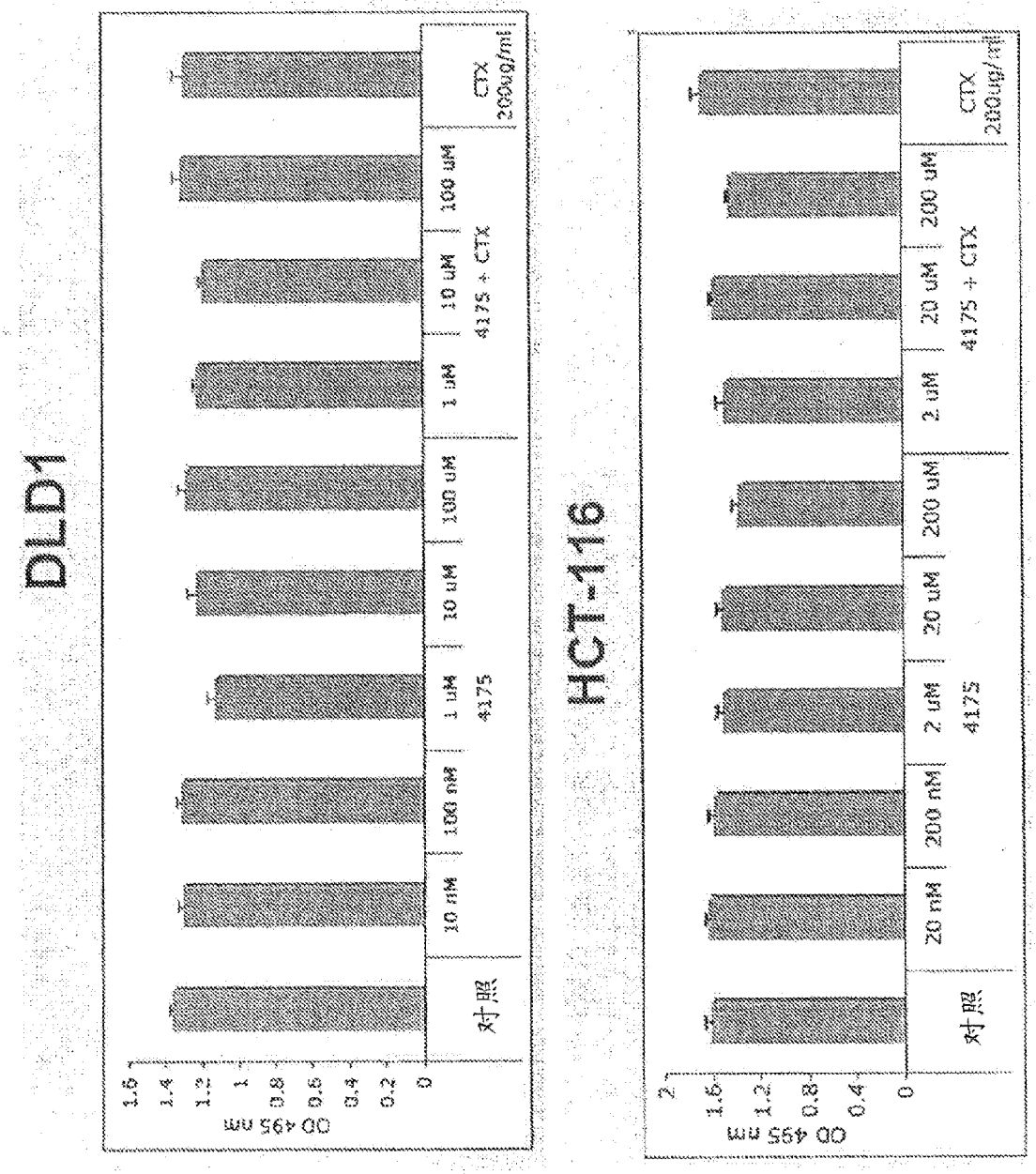

DLD1
OD 495 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
对照
10 nM
100 nM
1 uM
10 uM
100 uM
1 uM
10 uM
100 uM
4175
4175 + CTX
CTX 200ug/ml
HCT-116
OD 495 nm
2
1.6
1.2
0.8
0.4
0
对照
20 nM
200 nM
2 uM
20 uM
200 uM
2 uM
20 uM
200 uM
4175
4175 + CTX
CTX 200ug/ml

图 10

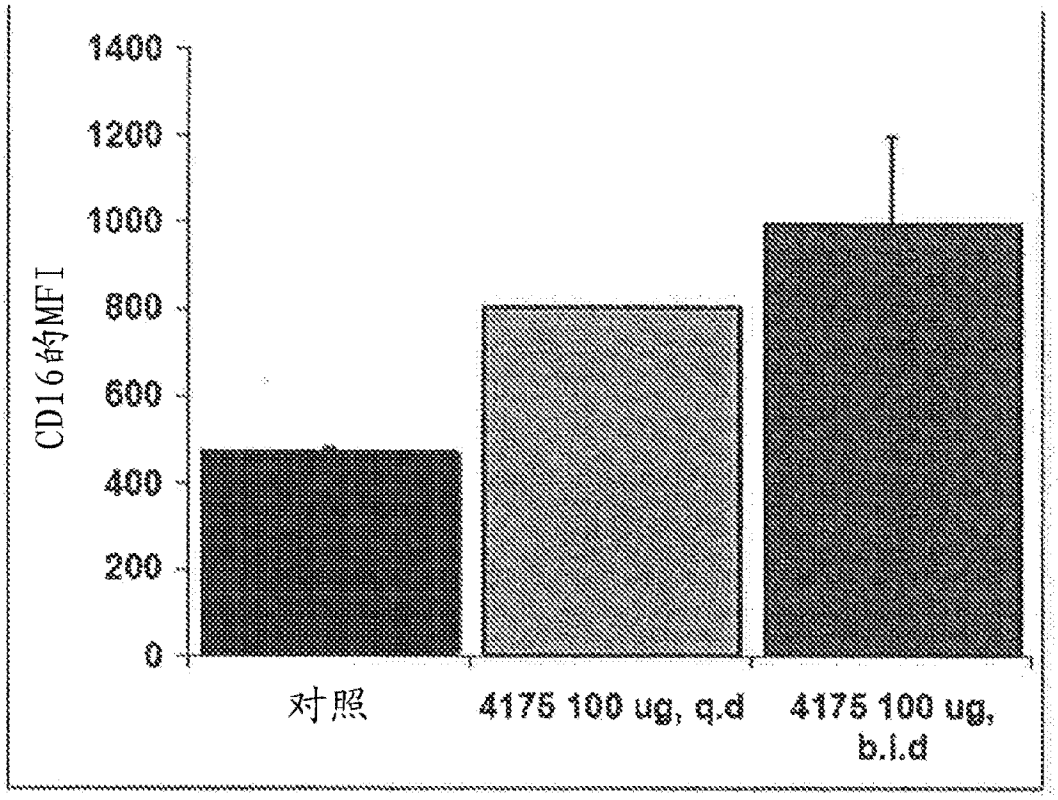

CD16的MFI
1400
1200
1000
800
600
400
200
0
对照
4175 100 ug, q.d
4175 100 ug, b.i.d

图 11

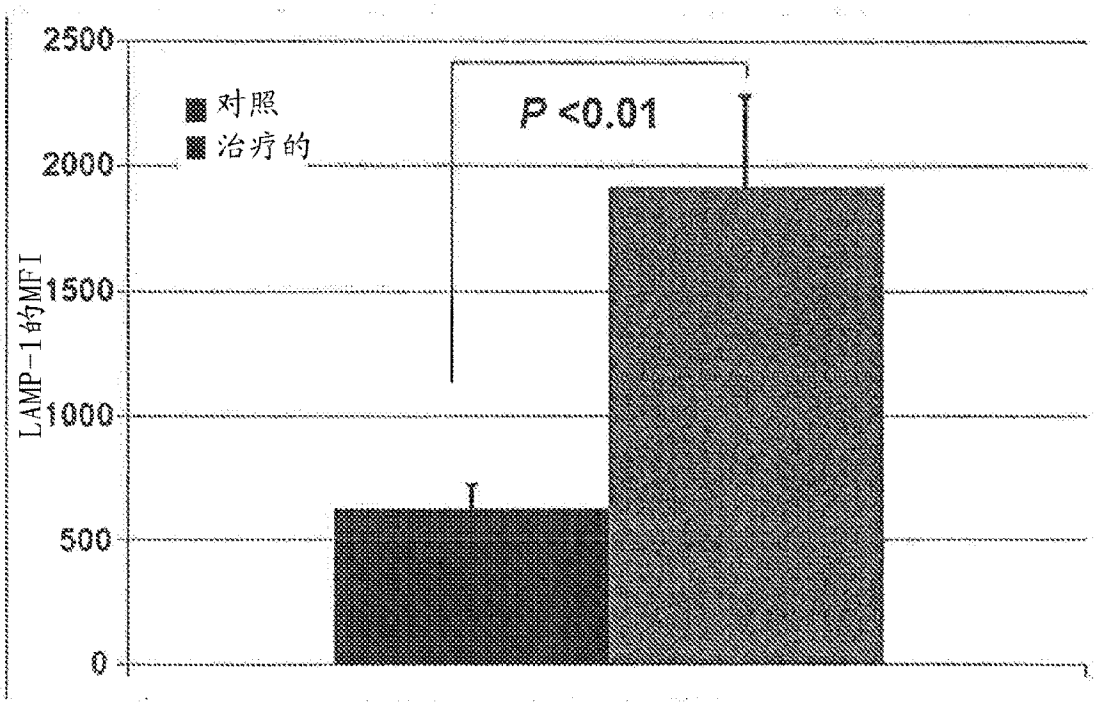


图 12

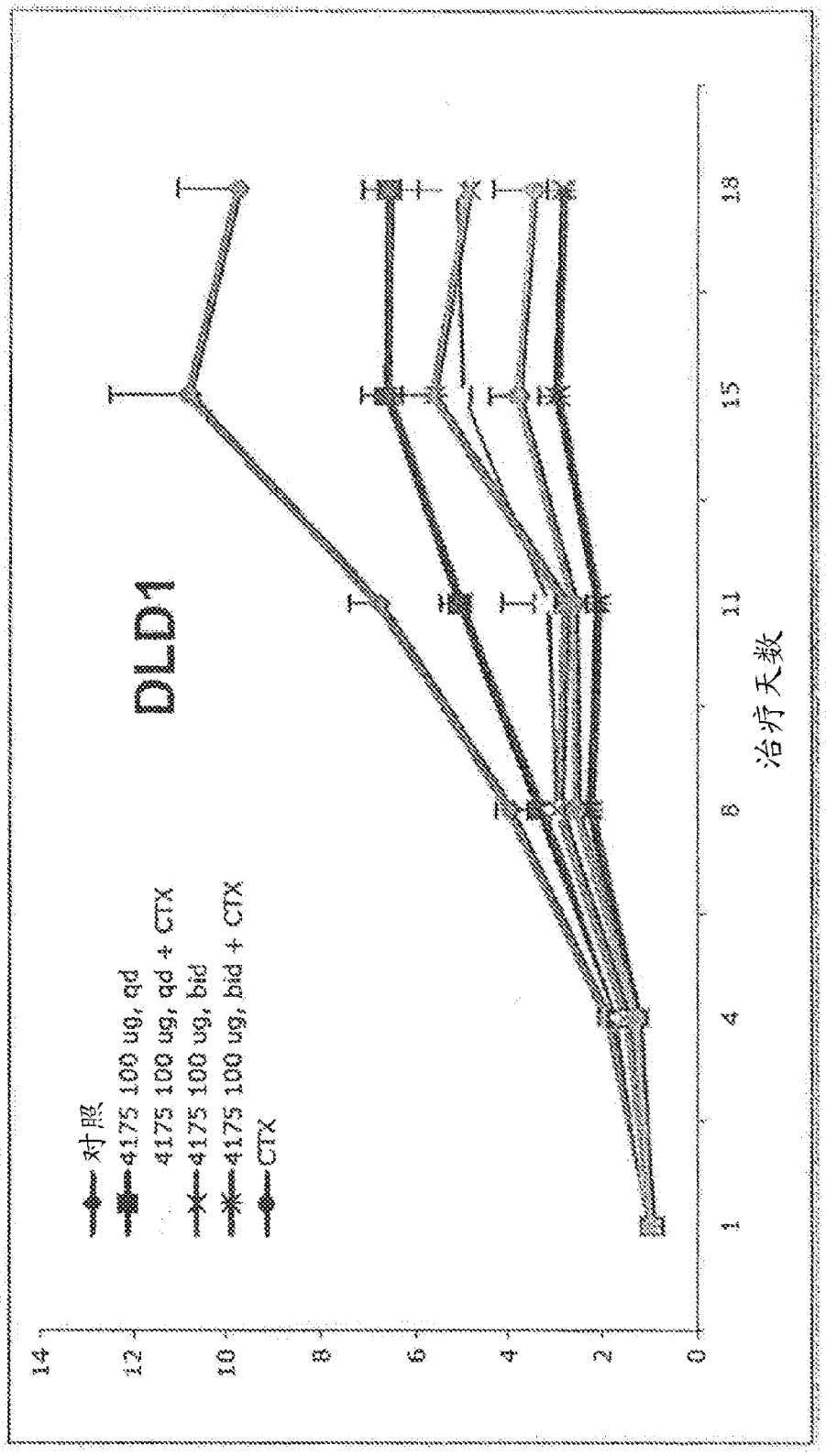

DLD1
对照
4175 100 ug, qd
4175 100 ug, qd + CTX
4175 100 ug, bid
4175 100 ug, bid + CTX
CTX
治疗天数
0
2
4
6
8
10
12
14
1
4
8
11
15
18

图 13A

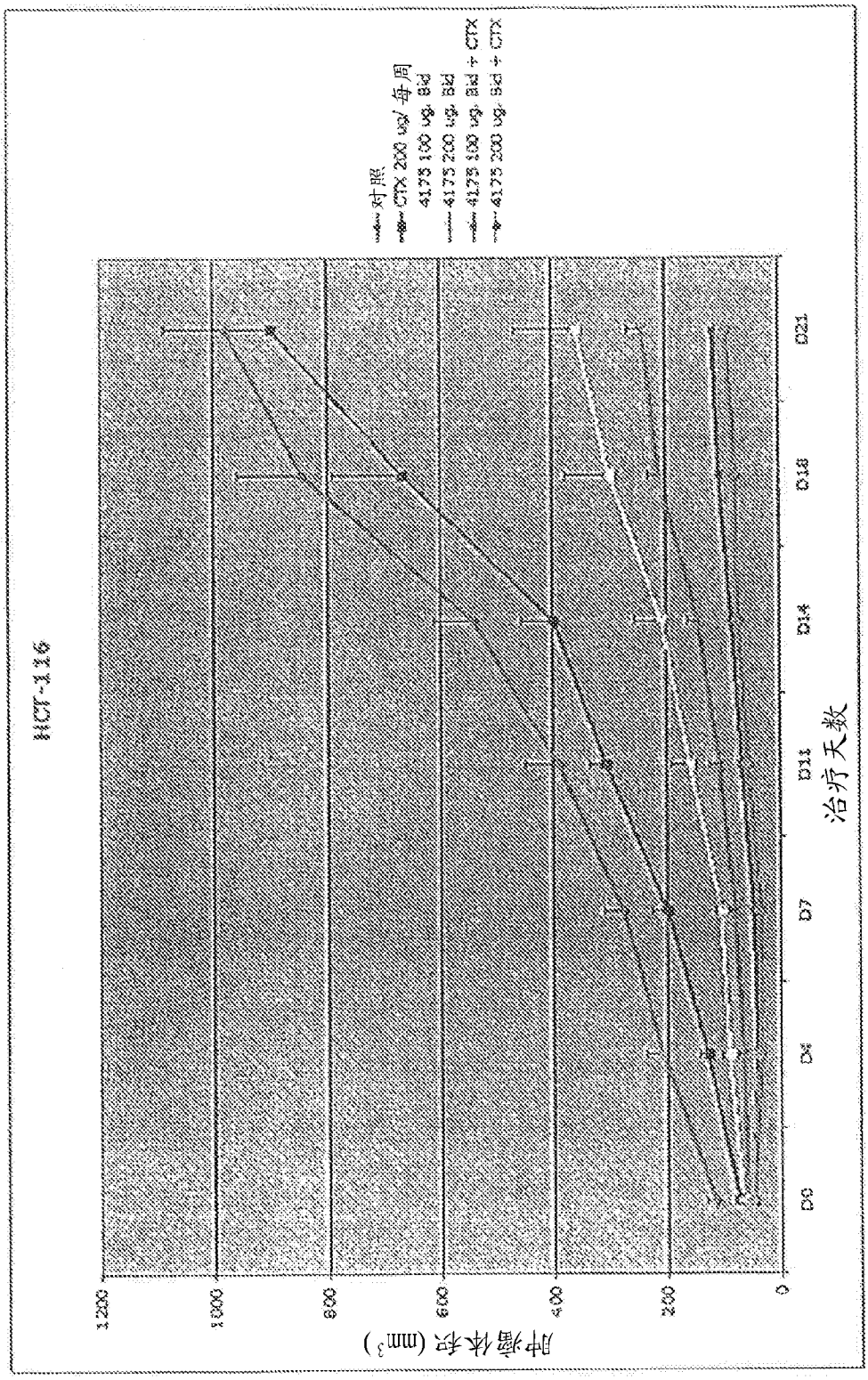

HCT-116
对照
CTX 200 mg/ 每周
4175 100 mg, Bid
4175 200 mg, Bid
4175 100 mg, Bid + CTX
4175 300 mg, Bid + CTX
肿瘤体积 (mm^3)
1200
1000
800
600
400
200
0
D0
D4
D7
D11
D14
D18
D21
治疗天数

图 13B

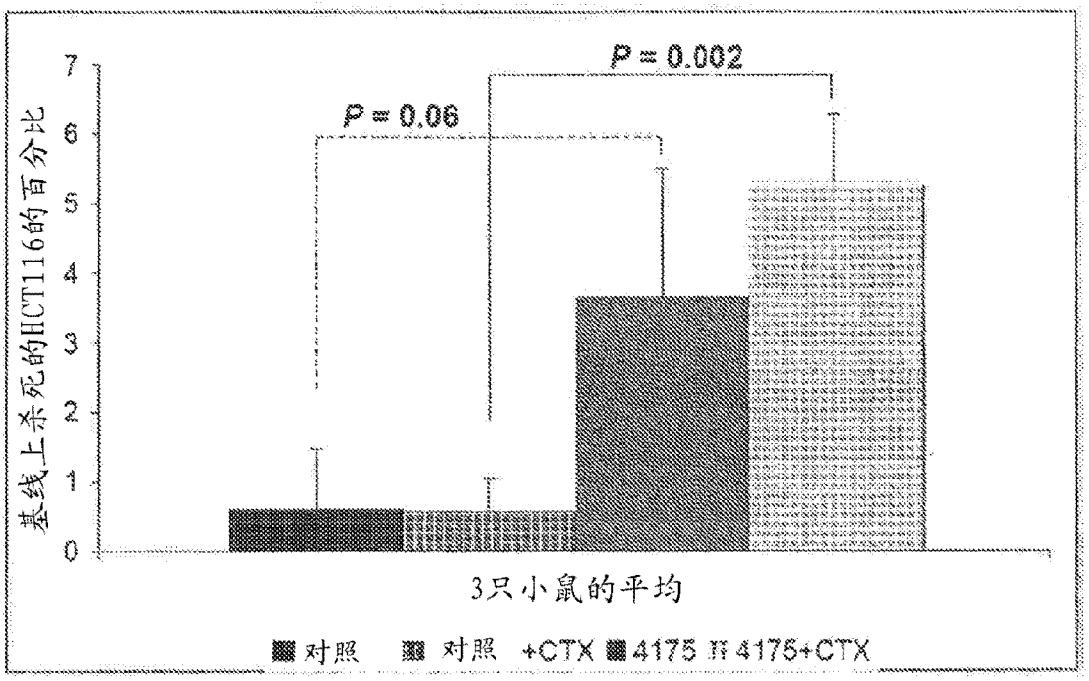

P = 0.002
P = 0.06
基线上杀死的HCT116的百分比
0
1
2
3
4
5
6
7
3只小鼠的平均
对照
对照 +CTX
4175
4175+CTX

图 14

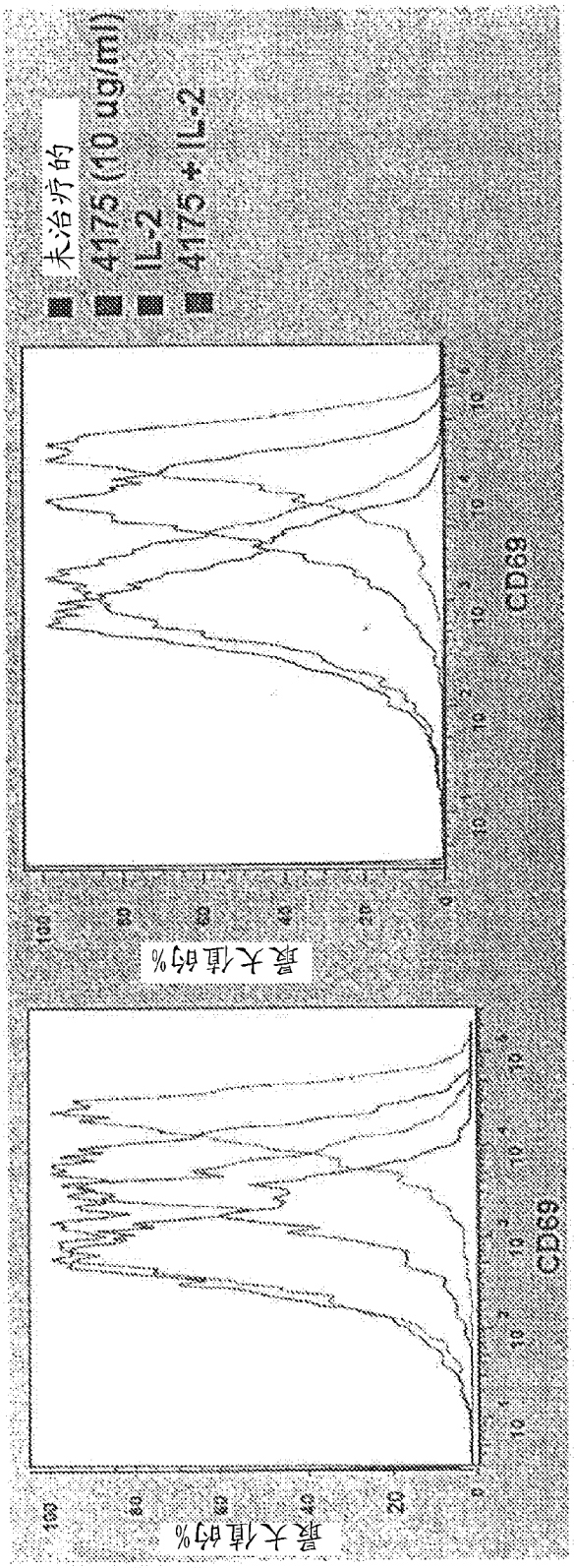


图 15

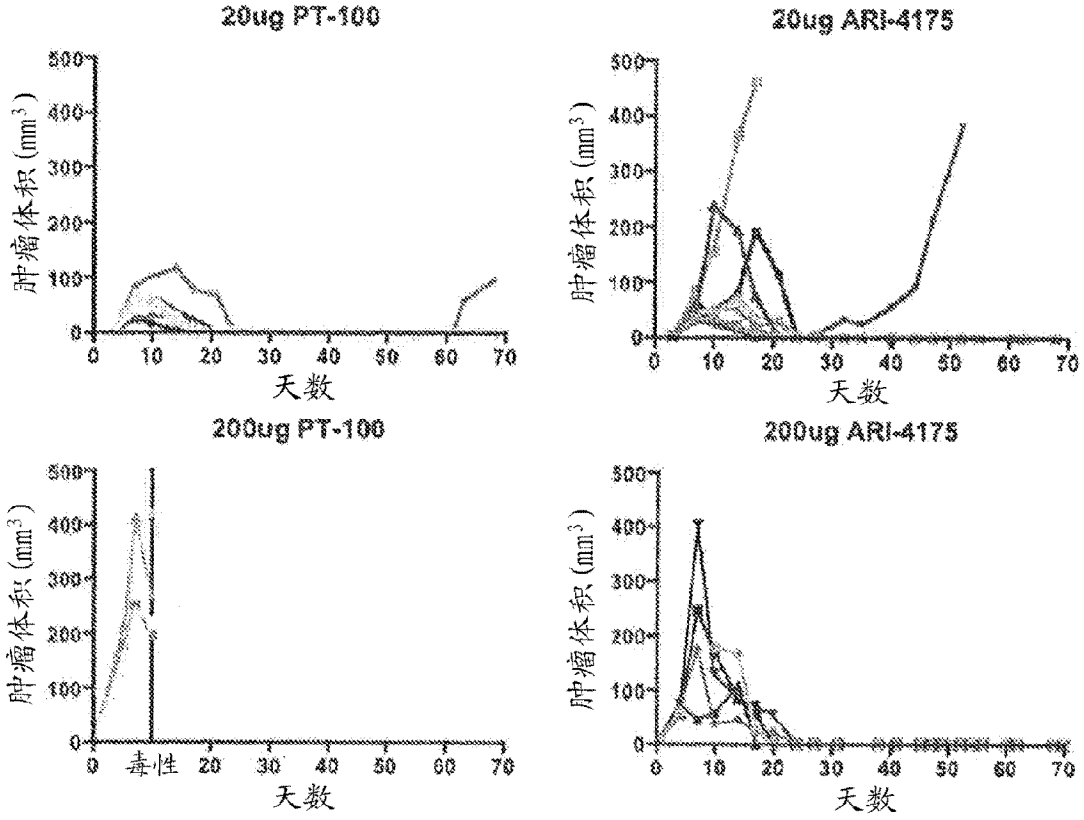

20ug PT-100
20ug ARI-4175
200ug PT-100
200ug ARI-4175
肿瘤体积 (mm3)
天数
毒性
0
100
200
300
400
500
10
20
30
40
50
60
70

图 16

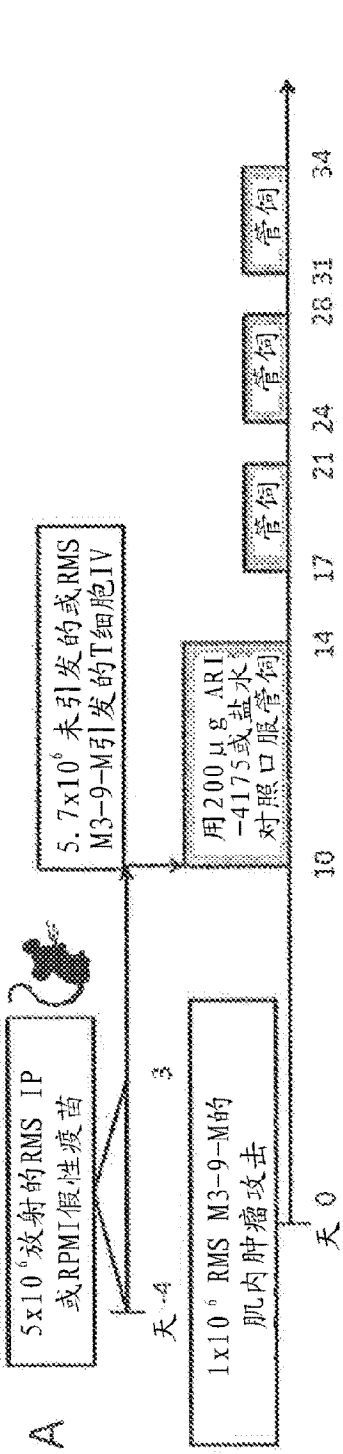

A
5x10⁶放射的RMS IP
或RPMI假性疫苗
1x10⁶ RMS M3-9-M的
肌内肿瘤攻击
5.7x10⁶未引发的或RMS
M3-9-M引发的T细胞IV
用200μg ARI
-4175或盐水
对照口服管饲
管饲
管饲
管饲
管饲
天-4
天0
3
10
14
17
21
24
28
31
34

图 17A

B
RMS M3-9-M
盐水
ARI-4175
ARI-4175+未引发的T细胞
ARI-4175+引发的T细胞
肿瘤体积 (mm³)
天数
存活率
天数

图 17B

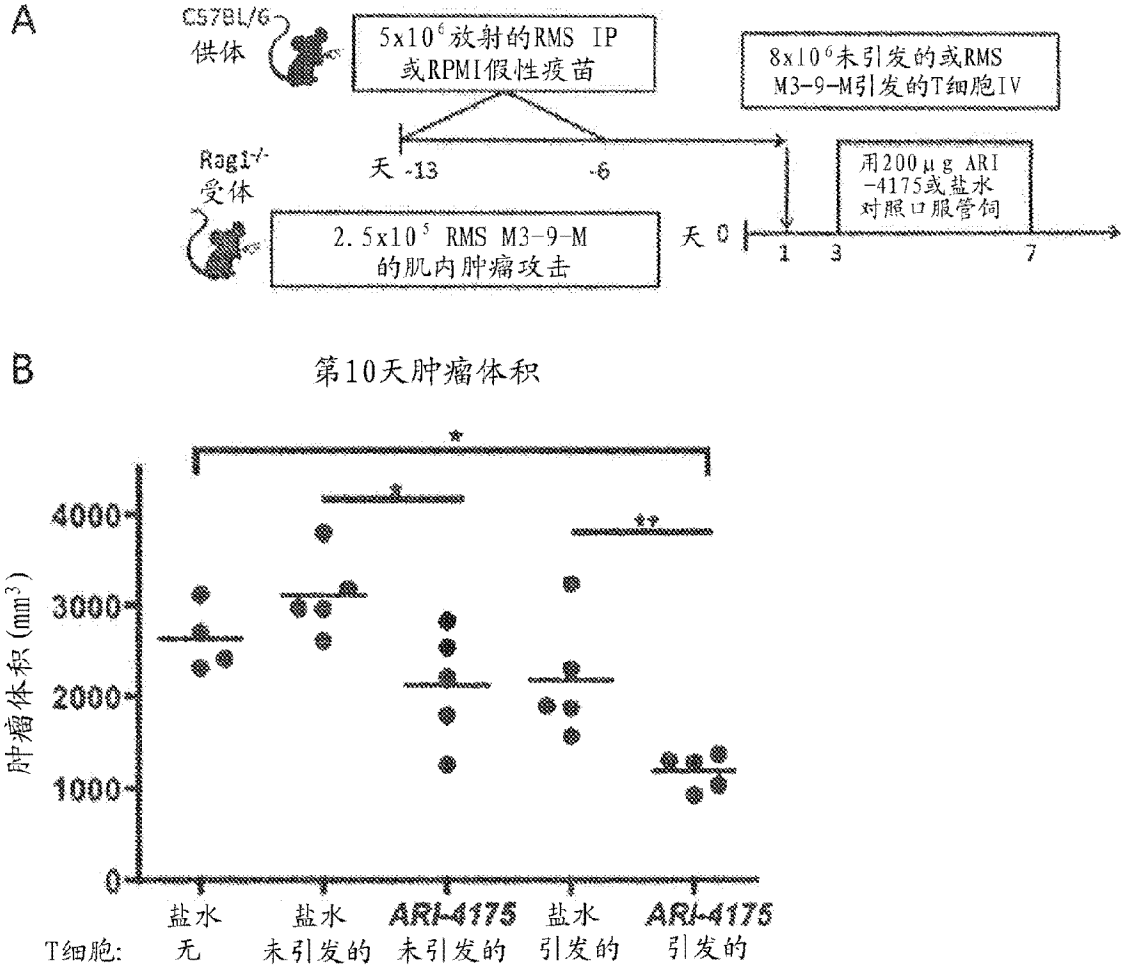

A
C57BL/6
供体
5x10⁶放射的RMS IP
或RPMI假性疫苗
8x10⁶未引发的或RMS
M3-9-M引发的T细胞IV
Rag1⁻/⁻
受体
天 -13
-6
用200μg ARI
-4175或盐水
对照口服管饲
2.5x10⁵ RMS M3-9-M
的肌内肿瘤攻击
天 0
1
3
7
B
第10天肿瘤体积
肿瘤体积 (mm³)
4000
3000
2000
1000
0
盐水
盐水
ARI-4175
盐水
ARI-4175
T细胞:
无
未引发的
未引发的
引发的
引发的

图 18

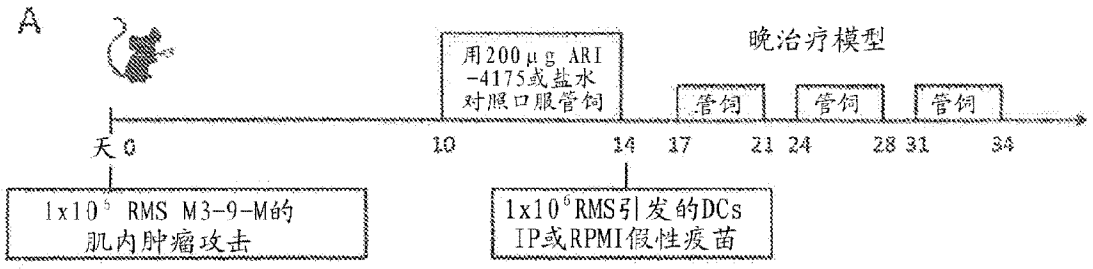

A
晚治疗模型
用200μg ARI -4175或盐水 对照口服管饲
管饲
管饲
管饲
天 0
10
14
17
21
24
28
31
34
1x10⁵ RMS M3-9-M的 肌内肿瘤攻击
1x10⁶RMS引发的DCs IP或RPMI假性疫苗

图 19A

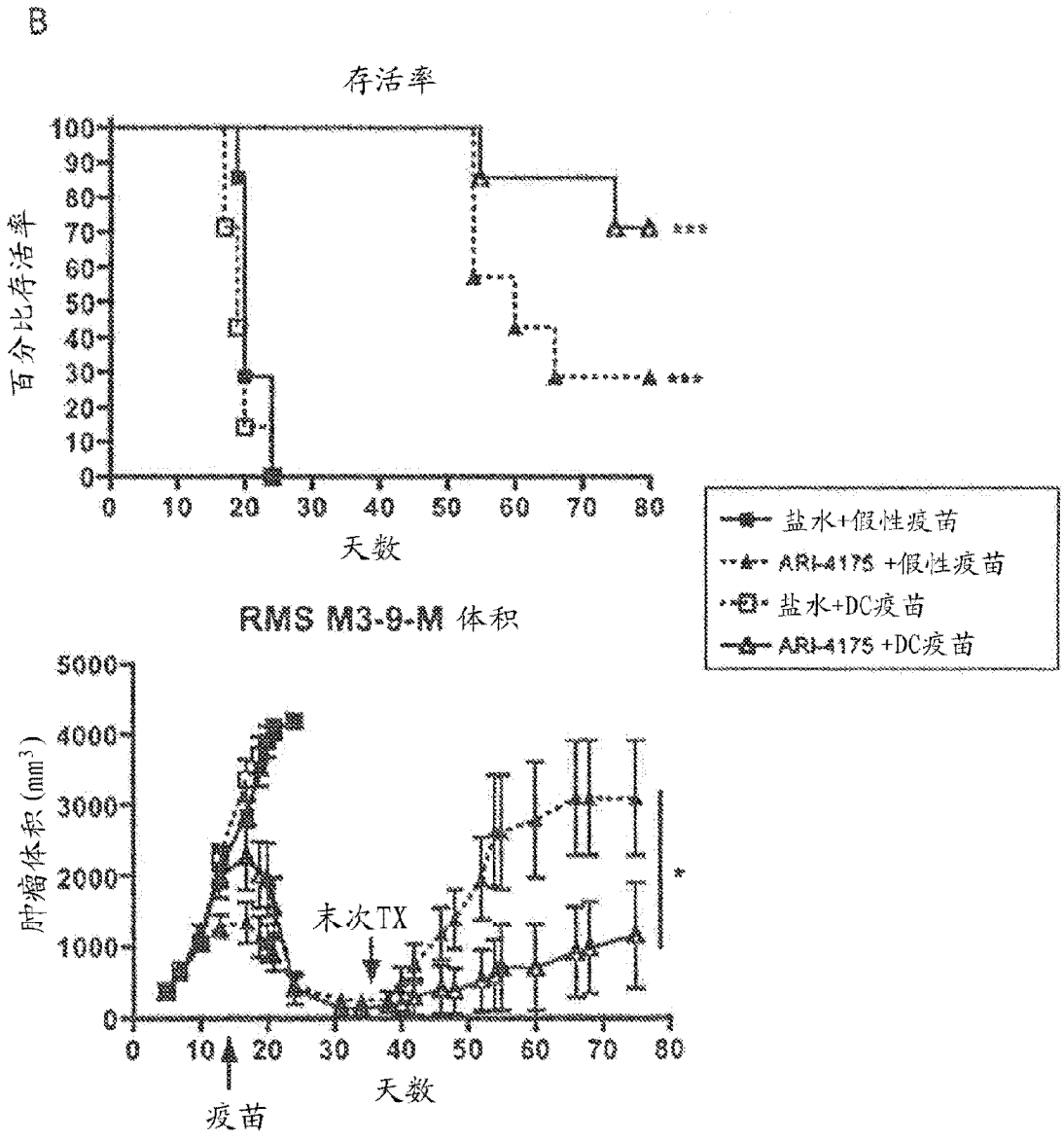
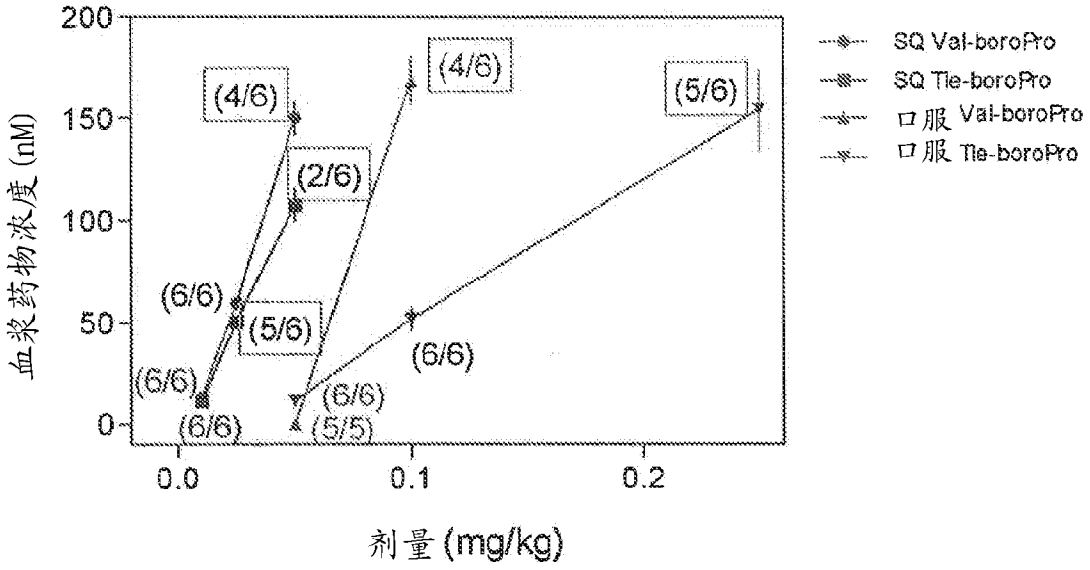

B
存活率
百分比存活率
天数
***
***
盐水+假性疫苗
ARI-4175 +假性疫苗
盐水+DC疫苗
ARI-4175 +DC疫苗
RMS M3-9-M 体积
肿瘤体积 (mm³)
末次TX
疫苗
天数
*

图 19B

SD大鼠中的MTD研究
给药后30 min采集的血浆

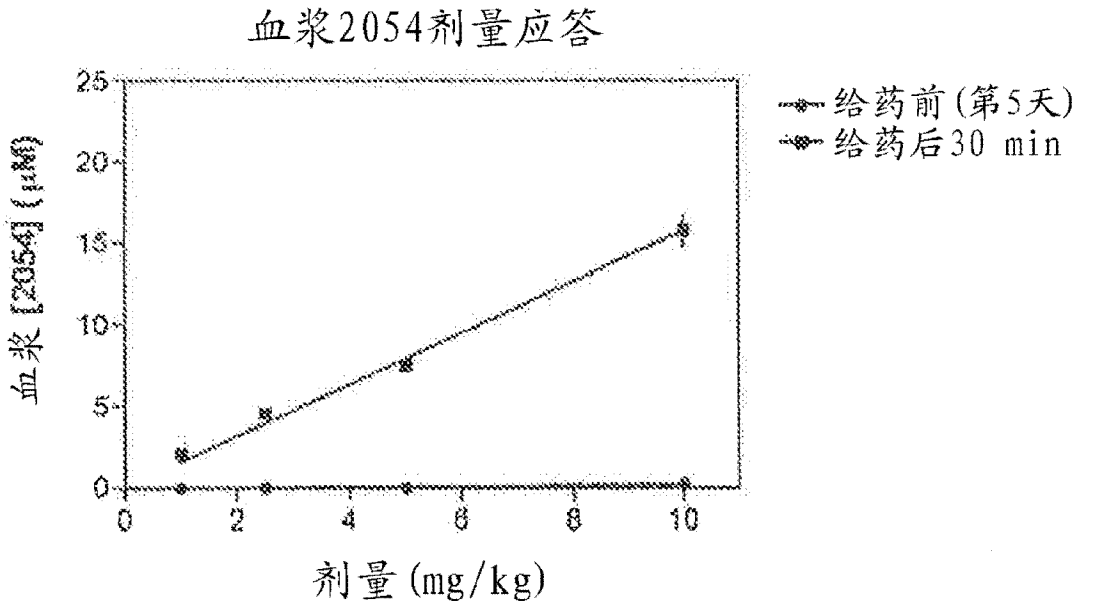


对于每次实验列出的存活率(存活数/每组总数)

药物浓度(nM)				
剂量 (mg/kg)	Val-bPro (SQ)	Tle-bPro (SQ)	Val-bPro (PO)	Tle-bPro (PO)
0.01	13.67 (0.64)	11.11 (0.19)		
0.025	59.58 (2.25)	**50.63 (1.41)**		
0.05	**150.54 (8.49)**	**107.80 (8.41)**	0.45 (0.56)	11.90 (1.82)
0.1			**168.44 (11.41)**	51.87 (5.30)
0.25				**154.74 (19.76)**

括号中为标准误差

图 20


血浆2054剂量应答
血浆 [2054] (μM)
25
20
15
10
5
0
0
2
4
6
8
10
剂量 (mg/kg)
给药前 (第5天)
给药后30 min

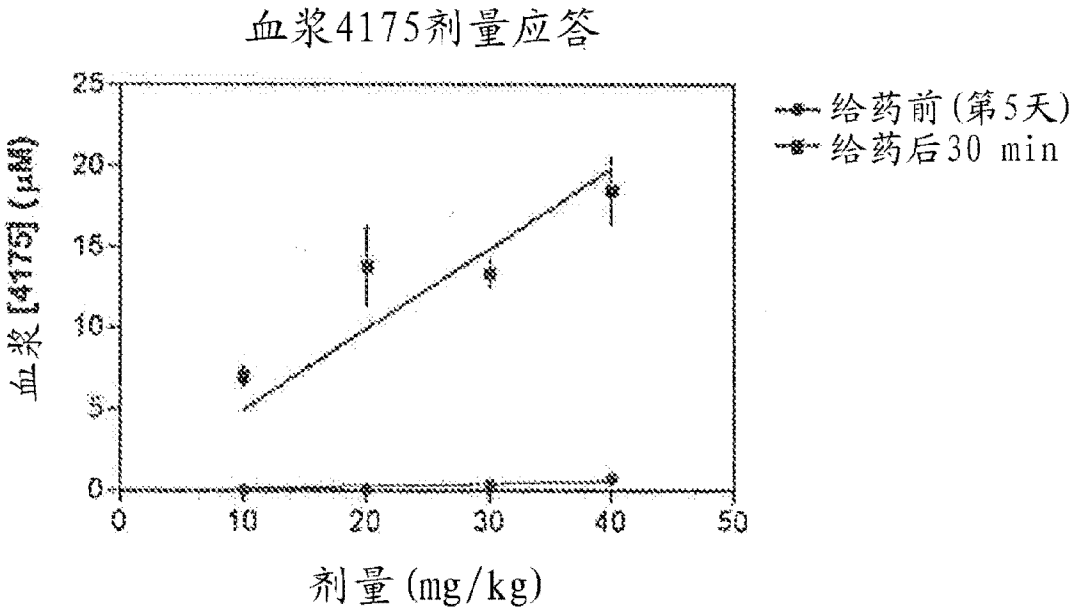
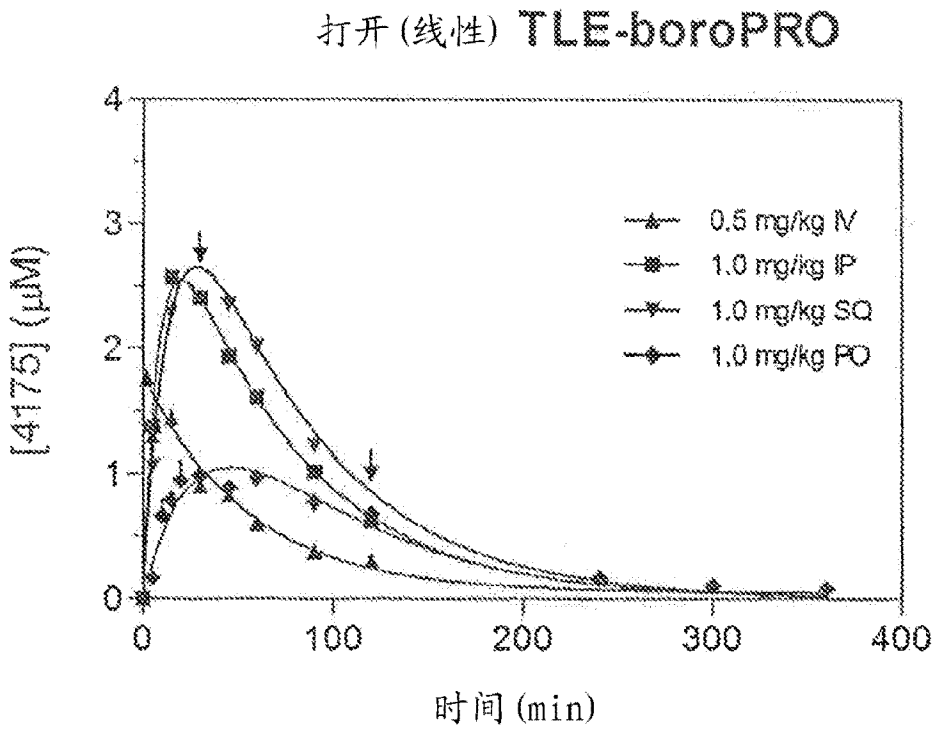

血浆4175剂量应答
血浆 [4175] (μM)
25
20
15
10
5
0
0
10
20
30
40
50
剂量 (mg/kg)
给药前 (第5天)
给药后30 min

图 21


打开(线性) TLE-boroPRO
[4175] (μM)
0.5 mg/kg IV
1.0 mg/kg IP
1.0 mg/kg SQ
1.0 mg/kg PO
0
1
2
3
4
0
100
200
300
400
时间(min)

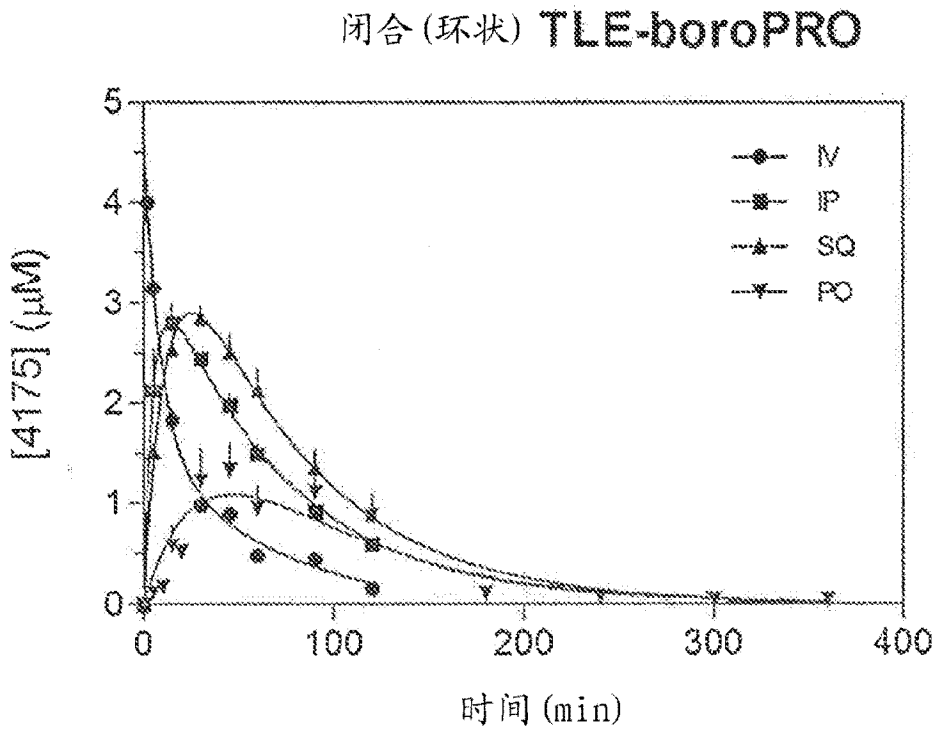

闭合(环状) TLE-boroPRO
[4175] (μM)
IV
IP
SQ
PO
0
1
2
3
4
5
0
100
200
300
400
时间(min)

图 22

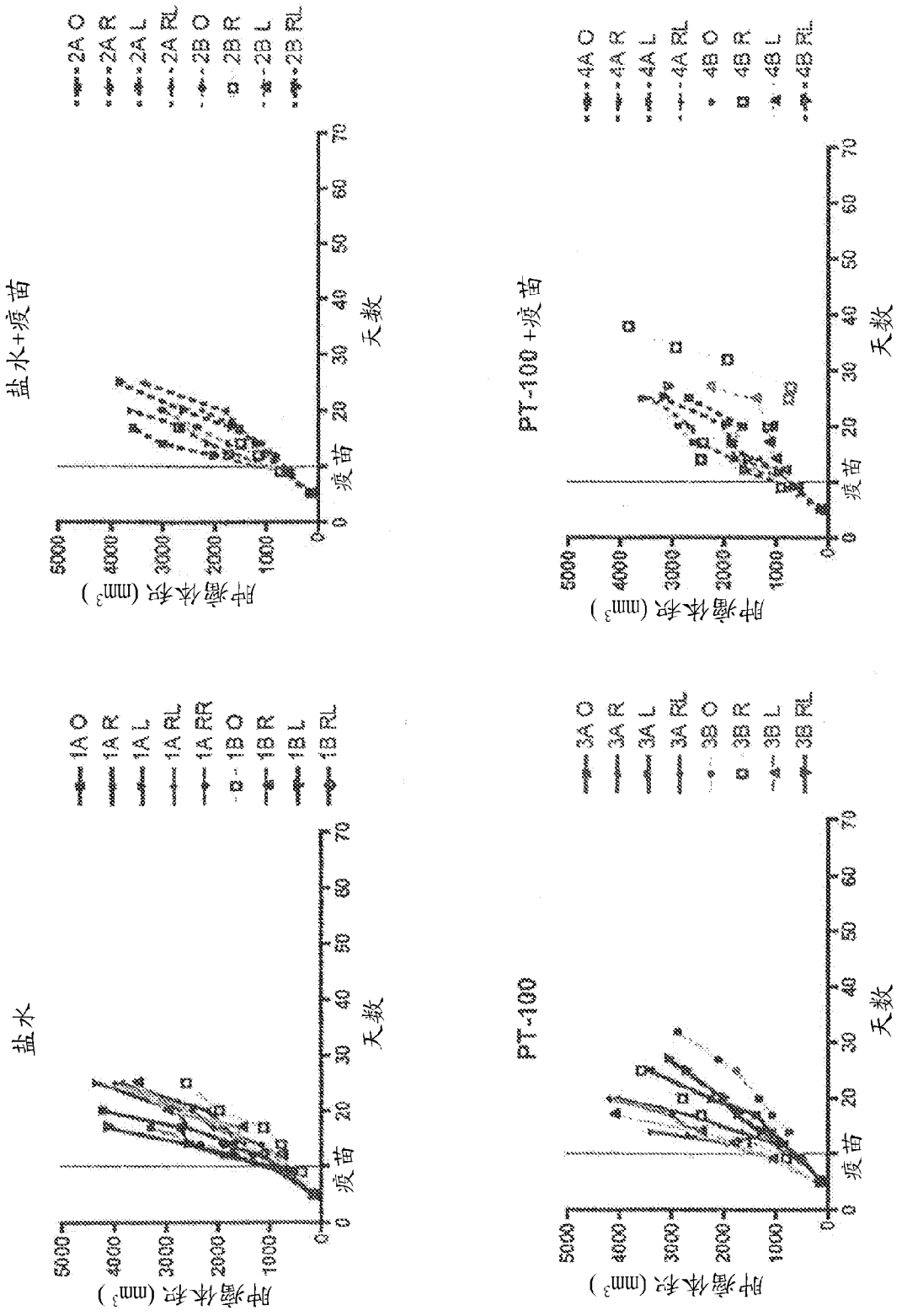

盐水
PT-100
盐水+疫苗
PT-100 +疫苗
肿瘤体积 (mm³)
天数
疫苗
1A O
1A R
1A L
1A RL
1A RR
1B O
1B R
1B L
1B RL
2A O
2A R
2A L
2A RL
2B O
2B R
2B L
2B RL
3A O
3A R
3A L
3A RL
3B O
3B R
3B L
3B RL
4A O
4A R
4A L
4A RL
4B O
4B R
4B L
4B RL

图 23

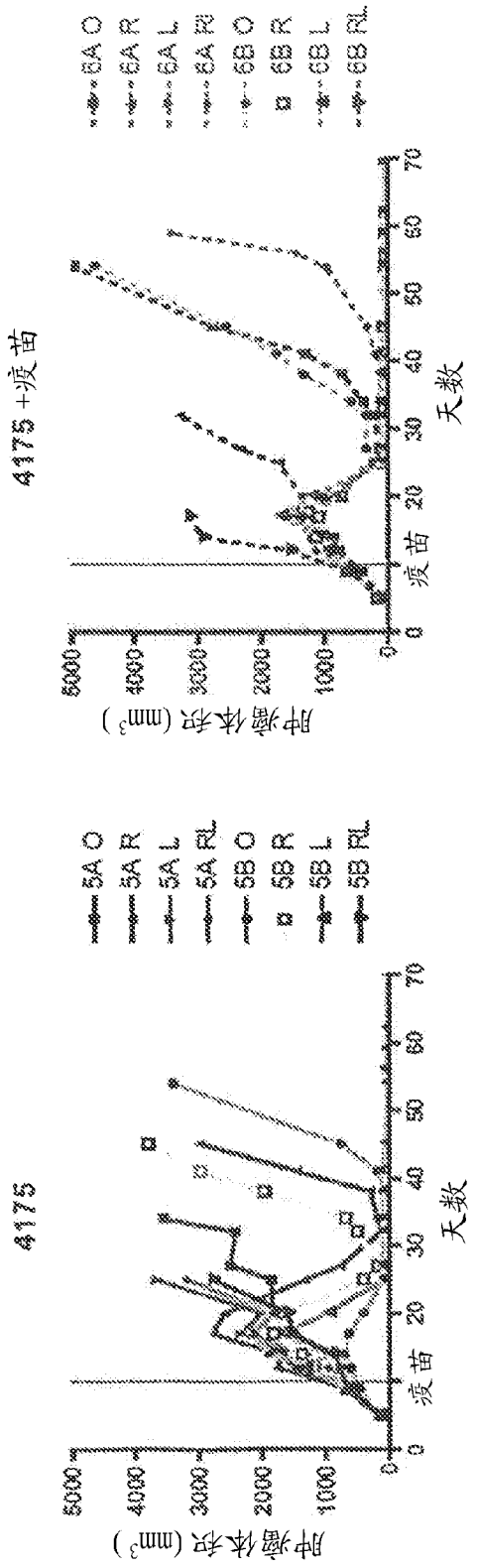

4175 +疫苗
肿瘤体积 (mm^3)
天数
疫苗
6A O
6A R
6A L
6A RL
6B O
6B R
6B L
6B RL
4175
肿瘤体积 (mm^3)
天数
疫苗
5A O
5A R
5A L
5A RL
5B O
5B R
5B L
5B RL

图 23 续