

Animal protocol

Lay description

Provide a lay abstract related to animals; specifically address (limit 2,500 characters/approx. one page)

- 1) the relevance of the work to the animal and/or human condition being studied
 - a. EBV/KSHV malignancies
- 2) the advancement of knowledge or the good to society
 - a. Ability to treat EBV/KSHV malignancies
- 3) the need to use animals in the research
 - a.
- 4) how the animals will be used
- 5) what species are required

Version 1

Human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV) are enveloped large DNA viruses which infect and establish lifelong latency in human B-cells. Nearly ubiquitous in human populations, EBV is the causative agent of infectious mononucleosis and is associated to a variety of B-cell malignancies including Hodgkin's lymphoma, Burkitt's Lymphoma, Nasopharyngeal Carcinoma and X-linked Lymphoproliferative syndrome. In immunodeficient populations, EBV is also associated with non-Hodgkin's lymphoma, and post-transplant lymphoproliferative disease.

KSHV is less widespread – its prevalence can reach as high as 50% depending on risk factors and geography. KSHV is the causative agent of Kaposi's sarcoma, which can present as one of four forms: Classic, Endemic, Organ Transplant associated, and Epidemic/AIDS-related. KSHV is also associated with Primary Effusion Lymphomas and Multicentric Castleman's disease.

Most chemotherapy/anti-cancer drugs are mitotic inhibitors which target fundamental cellular metabolic mechanisms such as microtubule assembly, protein synthesis, or DNA synthesis. As a result, therapeutic doses non-specifically inhibit all cellular growth, which results in significant and costly side effects both during and after treatment. While there are some drugs such as rituximab which are more specific to B-cells, they are not effective enough to be used alone. Considering the prevalence of latent EBV and KSHV infection, identifying small molecules which specifically target the state of EBV and KSHV viral infection will lead to better treatments against EBV and KSHV-associated malignancies. Specific small molecules are less likely to target basic metabolic mechanisms, and thus may be more efficient than current therapies while resulting in fewer side effects.

From a screen for small molecules which specifically inhibit the growth of EBV and KSHV-infected B-cell lines, we identified 3 specific growth inhibitors. Thiram and NSC#10010 are specific growth inhibitors of KSHV-infected cell lines, and Cellocidin is a specific growth inhibitor of EBV-infected cell lines.

What we intend to discover, is can these small molecule inhibitors effectively inhibit the proliferation of KSHV and EBV-associated lymphomas in vivo? Due to the complex molecular interactions between host and virus, an animal model is the most effective way to assess the effectiveness of a drug. It is the best first step towards determining if an effective in vitro drug, is as effective in vivo. To this end, we will inject immunocompromised NOD/SCID mice with human tumor cell lines. This will result in the eventual development of tumors. Upon the detection of tumors, we will treat each group of mice with one of the three inhibitors daily. We will then observe the mice daily to determine if the inhibitors improves the survivability of the mice. Afterwards, we will examine the tumors' molecular markers and determine if treatment had the intended effect and outcomes. If effective, these small molecule growth inhibitors can become one of the tools to be used against EBV and KSHV-associated malignancies.

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The prevalence of EBV and KSHV infection in human population along with ability to establish latency in its human hosts means that one therapeutic approach is to specifically target cells that are latently infected with EBV and KSHV.

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Version 1.1

Human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV) are enveloped large DNA viruses which infect and establish lifelong latency in human B-cells. Nearly ubiquitous in human populations, EBV is the causative agent of infectious mononucleosis and is associated to a variety of B-cell malignancies including Hodgkin's lymphoma, Burkitt's Lymphoma, Nasopharyngeal Carcinoma and X-linked Lymphoproliferative syndrome. In immunodeficient populations, EBV is also associated with non-Hodgkin's lymphoma, and post-transplant lymphoproliferative disease. KSHV is less widespread – its prevalence can reach as high as 50% depending on risk factors

and geography. KSHV is the causative agent of Kaposi's sarcoma, and is also associated with Primary Effusion Lymphomas and Multicentric Castleman's disease.

Most chemotherapy/anti-cancer drugs are mitotic inhibitors which target fundamental cellular metabolic mechanisms such as microtubule assembly, protein synthesis, or DNA synthesis. As a result, therapeutic doses non-specifically inhibit all cellular growth, which results in significant and costly side effects both during and after treatment. Considering the prevalence of latent EBV and KSHV infection, identifying small molecules which specifically target the state of EBV and KSHV viral infection will lead to better treatments against EBV and KSHV-associated malignancies. Specific small molecules are less likely to target basic metabolic mechanisms, and thus may be more efficient than current therapies while resulting in fewer side effects.

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Version 1.2, lay summary, Shortened for conciseness...1826 characters

Human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV) are viruses which infect and establish lifelong latency in human B-cells. Nearly ubiquitous in human populations, EBV is the causative agent of infectious mononucleosis and is associated to a variety of B-cell malignancies including Hodgkin's lymphoma and Burkitt's Lymphoma. KSHV is less widespread – its prevalence can reach as high as 50% depending on risk factors and geography. KSHV is the causative agent of Kaposi's sarcoma, and is also associated with Primary Effusion Lymphomas and Multicentric Castleman's disease. No drug treatments exist which are specific to EBV and KSHV-infected cells.

Considering the prevalence of latent EBV and KSHV infection, identifying small molecules which specifically target the state of EBV and KSHV viral infection will lead to better treatments against EBV and KSHV-associated malignancies. From our experiments in cell culture, we have identified 3 specific growth inhibitors. Thiram and NSC#10010 are specific growth inhibitors of KSHV-infected cell lines, and Cellocidin is a specific growth inhibitor of EBV-infected cell lines.

What we intend to discover, is can these small molecule inhibitors effectively inhibit the proliferation of KSHV and EBV-associated lymphomas in vivo? Due to the complex molecular interactions between host and virus, an animal model is the most effective way to assess the effectiveness of a drug. It is the best first step towards determining if an effective in vitro drug, is as effective in vivo. To this end, we will inject immunocompromised NOD/SCID mice with human tumor cell lines and treat them with the inhibitors. If effective, these small molecule growth inhibitors can become one of the tools to be used against EBV and KSHV-associated malignancies.

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Experimental setup:

Null control (20 mice)

EBV: LCL1 and LCL2 (20 each, + mock/treat = 80 mice)

KSHV: BC3 and BCBL1 (20 each, + mock/treat = 80 mice)

EBV/KSHV: JSC1 (20 mock/treat = 40 mice)

Control: Louckes and BJAB (20 each, + mock/treat = 80 mice)

240+60=300 mice

Drug dosages?

Research project description

PROVIDE A CLEAR DESCRIPTION OF THE OVERALL RESEARCH PROJECT, considering all of the procedures to be performed on the animals. DO NOT provide extensive details of individual procedures (e.g. surgery, behavioral studies) - such details should be provided in the Procedures section (limited to 15,000 characters/approx 10

pages):

-Include a description of the duration and endpoints of the study, including a timeline of all procedures performed (how they inter-relate) and the endpoints. Flow charts and tables may be uploaded as attachments using the "Upload Document" button below.

-Address specifically whether the procedures and/or administered compounds may adversely affect animal health or welfare and describe these effects in detail.

-Please note that "mortality" may not be used as an endpoint unless strong justification is provided of why "morbidity" cannot be used as an alternative.

Version 1

We want to determine if our three drugs would be good therapeutic agents against EBV/KSHV-induced lymphoma. In cell culture, we have found that the c-myc and NF-KB pathways are induced by these cell lines as part of its ability to trigger apoptosis or growth inhibition.

In these experiments, we will intra-peritoneally inject KSHV positive PEL cells into SCID mice. As a control, we will also inject KSHV and EBV negative BJAB and Louckes cells. Mice will be anesthetized with Ketamine/Xylazine. 100,000 viable cells in 100µL of calcium and magnesium free phosphate buffered saline will be injected intra-peritoneally on the back of the mice. Twice weekly measurements of the width and length of the primary tumor will be performed for each animal. When tumors grow to become visually apparent we will subcutaneously treat the mice with Thiram, NSC#10010 or Cellocidin. Cessation of the experiment will occur 3-4 months after the emergence of the primary tumor, and animals will be sacrificed prior to this point if they display any signs of distress (like mice becomes moribund, cachectic, develop ascities, skin ulcerations or are unable to obtain feed or water) or when the primary tumor reaches 20mm in diameter (or 10% of body weight). At autopsy, we will check the morphology of the treated and mock treated tumors and test the apoptotic markers. Hematoxylin and eosin stain sections will be prepared from the primary tumor, and all tumors will be tested for the expression of c-Myc and NF-KB. Any suffering experienced by the mice during any surgical procedure will be addressed by the administration of the anaesthetic Ketamine/Xylazine. To minimize any suffering and pain experienced by an animal during the course of the study, we will adhere to IACUC procedures for the euthanization of any animal that shows signs of distress.

These experiments will conclusively determine if these inhibitors have therapeutic potential against EBV and KSHV positive lymphomas.

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In our cell culture experiments, we showed

In these experiments, we will intra-peritoneally inject KSHV, EBV, KSHV/EBV, or non-virally infected positive PEL cells into SCID mice. As a control, we will also inject KSHV and EBV negative BJAB and Louckes cells. Mice will be anesthetized with Ketamine/Xylazine. 100,000 viable cells in 100µL of calcium and magnesium free phosphate buffered saline will be injected intra-peritoneally on the back of the mice. Twice weekly measurements of the width and length of the primary tumor will be performed for each animal. When tumors grow to become visually apparent we will subcutaneously treat the mice with Thiram, NSC#10010 or Cellocidin.

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Experimental setup from KSHV-ICN:

Null Control 20

BCBL1-KSHV infected B lymphoma cell line (Mock) 20

BCBL1-KSHV infected B lymphoma cell line (Treat) 20

JSC1-KSHV infected B lymphoma cell line (Mock) 20

JSC1-KSHV infected B lymphoma cell line (Treat) 20
LCL1-EBV transformed lymphoblastoid cell line (Mock) 20
LCL1-EBV transformed lymphoblastoid cell line (Treat) 20
LCL2-EBV transformed lymphoblastoid cell line (Mock) 20
LCL2-EBV transformed lymphoblastoid cell line (Treat) 20

Experimental setup v2:

Null Control 20
BCBL1-KSHV infected B lymphoma cell line (Mock) 20
BCBL1-KSHV infected B lymphoma cell line (Treat with Thiram) 20
BCBL1-KSHV infected B lymphoma cell line (Treat with NSC#10010) 20
BCBL1-KSHV infected B lymphoma cell line (Treat with Cellocidin) 20
BC3-KSHV infected B lymphoma cell line (Mock) 20
BC3-KSHV infected B lymphoma cell line (Treat with Thiram) 20
BC3-KSHV infected B lymphoma cell line (Treat with NSC#10010) 20
BC3-KSHV infected B lymphoma cell line (Treat with Cellocidin) 20
LCL1-EBV transformed lymphoblastoid cell line (Mock) 20
LCL1-EBV transformed lymphoblastoid cell line (Treat with Thiram) 20
LCL1-EBV transformed lymphoblastoid cell line (Treat with NSC#10010) 20
LCL1-EBV transformed lymphoblastoid cell line (Treat with Cellocidin) 20
LCL2-EBV transformed lymphoblastoid cell line (Mock) 20
LCL2-EBV transformed lymphoblastoid cell line (Treat with Thiram) 20
LCL2-EBV transformed lymphoblastoid cell line (Treat with NSC#10010) 20
LCL2-EBV transformed lymphoblastoid cell line (Treat with Cellocidin) 20

Version 1.12

We want to determine if our three drugs, Thiram, NSC#10010, and Cellocidin would be good therapeutic agents against EBV/KSHV-induced lymphomas in mice. In cell culture, we found that these three drugs are specific growth inhibitors of KSHV or EBV-infected cell lines. Additionally we found that the c-myc and NF-KB pathways are induced by these cell lines as part of its ability to trigger apoptosis or growth inhibition.

In these experiments, we will intra-peritoneally inject KSHV or EBV infected positive PEL cells into SCID mice. Mice will be anesthetized with Ketamine/Xylazine. 100,000 viable cells in 100µL of calcium and magnesium free phosphate buffered saline will be injected intra-peritoneally on the back of the mice. Twice weekly measurements of the width and length of the primary tumor will be performed for each animal. When tumors grow to become visually apparent we will intra-peritoneally treat the mice with Thiram, NSC#10010 or Cellocidin.

Cessation of the experiment will occur 3-4 months after the emergence of the primary tumor, and animals will be sacrificed prior to this point if they display any signs of distress (like mice becomes moribund, cachectic, skin ulcerations or are unable to obtain feed or water) or when the primary tumor reaches 20mm in diameter (or 10% of body weight). At autopsy, we will check the morphology of the treated and mock treated tumors and test the apoptotic markers. Hematoxylin and eosin stain sections will be prepared from the primary tumor, and all tumors will be tested for the expression of c-Myc and NF-KB. Any suffering experienced by the mice during any surgical procedure will be addressed by the administration of the anaesthetic Ketamine/Xylazine. To minimize any suffering and pain experienced by an animal during the course of the study, we will adhere to IACUC procedures for the euthanization of any animal that shows signs of distress.

These experiments will conclusively determine if these inhibitors have therapeutic potential against EBV and KSHV positive lymphomas.

(how the heck do you weigh a tumor?)

There are no known alternative methods that can be utilized to address the question of KSHV mediated tumorigenesis in vivo. The proposed animal work is the logical progression of the in vitro work already completed.

In Pubmed, "Animal testing alternative KSHV" returned Zero hits. A search for KSHV animal model yielded 77 hits. Most of the 77 hits were irrelevant; and those that were relevant, use the same NOD/SCID animal model that we will use.

In Altweb, "Animal testing alternative KSHV" returned Zero hits, as did a search for KSHV animal model.

In addition, the investigators are intimately familiar with the latest developments in the herpesvirus field and would like to assure the panel that an alternative technique is not available.

Animals must be used in this research because there is no other way to study the effects of these drugs in an in vivo system. If any of these drugs have a particularly strong effect on herpesvirus-infected cells in vivo, it will identify promising drug

candidates for EBV and KSHV-associated malignancies. Computer models cannot be used due to the complexity of the molecular mechanisms to be examined.

SCID mice are B and T cell deficient mice that provide a unique model for the progression of human disease. They are relatively easy to use and have been well characterized and utilized as a suitable animal model for the determination of drug effects on cancer cell lines in vivo.

A lower phylogenetic species cannot be used due to the lack of an immune system in lower phylogenetic species.

BCBL₁ and JSC₁ groups are KSHV-infected, so they're the two primary groups in the experiment. LCL₁ and LCL₂ cell lines are EBV-infected. EBV is another member of the gammaherpesvirus family which KSHV is also a member of, so the LCL₁ and LCL₂ groups are controls for viral infection, and for the specificity of the gamma secretase treatment against KSHV.

BCBL₁ and BC₃ groups are KSHV-infected, LCL₁ and LCL₂ cell lines are EBV-infected. These four cell lines are the primary groups in the experiment. EBV and KSHV are both members of the gammaherpesvirus family. Thiram and Cellocidin are specific to EBV, so the KSHV-infected cell lines are the control for EBV-infected cell lines. NSC#10010 is specific to KSHV, so EBV-infected cell lines are the control for KSHV-infected cell lines. This experimental setup should establish the specificity and efficacy of the drugs.

October 9, 2012

After the formation of a tumor, the animals will be injected with 1.1mg/mouse of a single inhibitor daily. We will alternate sides on the abdomen. Twice a week, they will be examined more closely, monitoring for body condition score, dimensions and location of the primary tumor, and the weight of the animal. Cessation of the experiment will occur 3-4 months after the emergence of the primary tumour, and animals will be sacrificed prior this point if any of the following occur: they reach a body condition score of 2/5, they display any signs of distress (diarrhea, prolapsed rectum, weight loss), when the primary tumour reaches 20mm in diameter or 120% of initial body weight, the animals body condition score, a single tumor exceeds 0.8cm³, multiple tumors exceed 1.2cm³. Ulceration is not expected to occur at the tumor site, but if it occurs over greater than 25% of the tumor surface area, the experiment will be stopped.

At autopsy, hematoxylin and eosin stain sections will be prepared from the primary tumour. All tumours will be tested for the expression of c-Myc and NF-kB. These experiments will provide evidence for whether or not these growth inhibitors have therapeutic potential against EBV or KSHV positive lymphomas.

<>Literature search

There are no known alternative methods that can be utilized to address the question of KSHV mediated tumorigenesis in vivo. The proposed animal work is the logical progression of the in vitro work already completed.

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The addition of the words "Pain", "Distress", "mouse", "mice", and "Tumor" returned results that were a subset of the above searches

In Pubmed, "Animal testing alternative EBV" returned two hits, both of which were irrelevant (one is a mouse breeding paper, the other is a cattle breeding paper). A search for EBV animal model yielded 516 hits. Most of the hits were irrelevant because EBV also stands for "estimated breeding values (EBV)"; and those that were relevant, use the same NOD/SCID xenograft animal model that we will use.

In Altweb, "Animal testing alternative EBV" returned zero relevant hits, as did a search for EBV animal model.

The addition of the words "Pain", "Distress", "mouse", "mice", and "Tumor" returned results that were a subset of the above searches

In addition, the investigators are intimately familiar with the latest developments in the herpesvirus field and would like to assure the panel that an alternative technique is not available.

How will you reconcile therapeutic dosage? I guess that should be part of the actual experiment, but not described in the protocol...

An in vivo system will also show us whether or not a drug is deactivated by a cellular process... Drug scheduling also... is there any way to determine the drug half-life in an animal model?

Additions:

Signs of ascites, peritoneal cavity, and subcutaneous tumor formation were detected at weeks 8–22 after transplantation in the majority but not all mice receiving BCBL-1 cells by any route (groups 1-4).

-from Picchio BCBL-1 and ascities

(Picchio GR, et al. The KSHV/HHV8-infected BCBL-1 lymphoma line causes tumors in scm mice but fails to transmit virus to a human peripheral blood mononuclear cell graft. *Virology* 1997; 238:22-9).

We have reviewed IACUC guide 2, and will monitor mice once a week until the appearance of ascities. After the appearance of ascities, we will monitor the health of the animals twice a week. Following IACUC guidelines (body weight, body condition score, tumor dimensional criteria/anatomy, etc.).

IACUC guidelines on tumors and ascities: Things to pay attention to in regards to:

- 1) Tumors
 - a. Must be monitored weekly before tumor is detected
 - b. Must be monitored twice a week after the tumor is detected, more frequently as necessary based on the condition of the mouse and speed of the tumor.
 - c. Follow the IACUC guideline for body score and euthanasia
 - d. We do not expect multiple tumors
- 2) Ascities
 - a. In our experiment, when body weight exceeds 120% of normal, rodents will be euthanized
 - b. Pressure will be relieved before distention causes discomfort or interferes with normal activity

Abstract:

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Version 1.3, lay summary, Shortened for conciseness...1826 characters

Human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV) are viruses which infect and establish lifelong latency in human B-cells. Nearly ubiquitous in human populations, EBV is the causative agent of infectious mononucleosis and is associated to a variety of B-cell malignancies including Hodgkin's lymphoma and Burkitt's Lymphoma. KSHV is less widespread – its prevalence can reach as high as 50% depending on risk factors and geography. KSHV is the causative agent of Kaposi's sarcoma, and is also associated with Primary Effusion Lymphomas (PEL) and Multicentric Castleman's disease. No drug treatments exist which are specific to EBV and KSHV-infected cells.

Considering the prevalence of latent EBV and KSHV infection, identifying small molecules which specifically target the state of EBV and KSHV viral infection will lead to better treatments against EBV and KSHV-associated malignancies. From our experiments in cell culture, we have identified 3 specific growth inhibitors. Thiram and NSC#10010 are specific growth inhibitors of KSHV-infected cell lines, and Cellocidin is a specific growth inhibitor of EBV-infected cell lines.

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Version 1.13

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In these experiments, we will intra-peritoneally inject KSHV or EBV infected positive PEL cells into SCID mice. Mice will be anesthetized with Ketamine/Xylazine. 100,000 viable cells in 10uL of calcium and magnesium free phosphate buffered saline will be injected intra-peritoneally on the back of the mice. Once subcutaneous tumors and/or ascities form, we will observe them twice a week and begin to treat the mice.

Twice weekly measurements of the width and length of the primary/largest tumor will be performed for each animal. When tumors grow to become visually apparent we will intra-peritoneally treat the mice with Thiram, NSC#10010 or Cellocidin. Animals will be monitored in accordance to the IACUC guideline on Rodent Tumor Production. Animals will be evaluated by body score and tumor measurements. If body score falls to 1 or to 2 with decreased activity/responsiveness, it will be euthanized. If the animal displays any signs of distress (like mice becomes moribund, cachectic, skin ulcerations or are unable to obtain feed or water), when the primary tumor reaches 20mm in diameter (or 10% of body weight), when the maximum volume of all tumors exceeds 1.2 cubic cm, or if the weight of the mouse with ascities exceeds 120% of initial weight.

Cessation of the experiment will occur 3-4 months after the emergence of a primary tumor. Animals will be sacrificed prior to this point if they display any conditions requiring euthanasia based on the IACUC rodent tumor production guideline.

At autopsy, we will check the morphology of the treated and mock treated tumors and test the apoptotic markers. Hematoxylin and eosin stain sections will be prepared from the primary tumor, and all tumors will be tested for the expression of c-Myc and NF- κ B. Any suffering experienced by the mice during any surgical procedure will be addressed by the administration of the anaesthetic Ketamine/Xylazine. To minimize any suffering and pain experienced by an animal during the course of the study, we will adhere to IACUC procedures for the euthanization of any animal that shows signs of distress. These experiments will determine if these inhibitors have therapeutic potential against EBV and KSHV positive lymphomas.

Explanation letter:

All requested modifications were made.

For clarification:

1) Experimental design: The primary tumor is the largest tumor that is noticeable. We do not know ahead of time if that primary tumor will be peritoneal or subcutaneous, or if ascities will develop. We can only observe for subcutaneous tumors and ascities. As they occur, we will follow the relevant IACUC guideline on Rodent Tumor Production.

2) Procedure: we will use ketamine/xylazine to anesthetize the mice for tumor injection. Also, we will not be removing the ascites if they occur. If ascities occurs and the body weight exceeds 120% of the initial weight before the development of ascities, then we will euthanize the mice.