(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 July 2003 (24.07.2003)

PCT

(10) International Publication Number
WO 03/059334 A2

(51) International Patent Classification[7]: A61K 31/00

(21) International Application Number: PCT/US02/31570

(22) International Filing Date: 15 October 2002 (15.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/356,623 25 October 2001 (25.10.2001) US

(71) Applicant *(for all designated States except US)*: **ELI LILLY AND COMPANY** [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **GLASS, John, Irvin** [US/US]; 625 Bryn Mawr Drive, Indianapolis, IN 46260 (US).

(74) Agents: **MCGRAW, Elizabeth, A.** et al.; ELI LILLY AND COMPANY, P. O. Box 6288, Indianapolis, IN 46206-6288 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designation*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/059334 A2

(54) Title: GEMCITABINE IN THE TREATMENT OF SMALLPOX

(57) Abstract: A method of treating smallpox in a mammalian patient in need thereof comprising administering a therapeutically effective dose of gemcitabine to the patient.

GEMCITABINE IN THE TREATMENT OF SMALLPOX

It has been known for some time that antiviral drugs can be found among the general family of nucleosides. Gemcitabine is one such compound which was first described in US patent #4,808,614, hereinafter incorporated by reference, as an antiviral agent, specifically designed as a "potent agent against herpes virus." Herpes viruses are DNA viruses that often cause blistery lesions in the skin and mucous membranes. Herpes viruses exist in latent and actively replicating forms. Herpes virus is a members of three DNA virus sub-families: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae.

Like the herpes virus, small pox is a DNA virus that is infectious and contagious through human contact. However, unlike the herpes virus, smallpox is a highly contagious and sometimes fatal infectious disease. There is no specific treatment for smallpox disease, and the only prevention is vaccination. The name is derived from the Latin word for "spotted" and refers to the raised bumps that appear on the face and body of an infected person. Two clinical forms of smallpox have been described. Variola major is the severe form of smallpox, with a more extensive rash and higher fever. It is also the most common form of smallpox. There are four types of variola major smallpox: ordinary (the most frequent); modified (mild and occurring in previously vaccinated persons); flat; and hemorrhagic. Historically, variola major has a case-fatality rate of about 30%. However, flat and hemorrhagic smallpox, which are uncommon types of smallpox, are usually fatal. Hemorrhagic smallpox has a much shorter incubation period and is likely not to be initially recognized as smallpox when presenting to medical care. Smallpox vaccination also does not provide much protection, if any, against hemorrhagic smallpox. Variola minor is a less common clinical presentation, and much less severe disease (for example, historically, death rates from variola minor are 1% or less). Small pox belongs to the sub-family Chordopoxvirinae of the genus orthopoxvirus.

Orthopoxviruses, most notably smallpox virus, pose major risks to human health. Smallpox virus had killed tens of millions of people and disfigured countless millions more by the time it was declared eradicated in the 1970s[1]. As a result of the eradication of smallpox and because of the small risk of morbidity or mortality associated with vaccination against smallpox, the vaccination program that had wiped smallpox from the

face of the earth was discontinued. Consequently most humans are now vulnerable to smallpox infection, and it is now feared that variola virus, the agent of smallpox, might be used as an agent of bioterrorism[2]. Other orthopox viruses that pose potential health risks include monkeypox, which causes a disease that resembles smallpox although is less lethal[3], and camelpox, which is feared as a possible agent of bioterrorism[4]. Furthermore, a skin infection of AIDS patients called molluscum contagiosum is caused by another poxvirus[5]. Finally, vaccinia virus, which is used to vaccinate humans against smallpox, can cause severe infections in immunocompromised recipients. Thus widespread renewal of smallpox vaccination would result in life threatening complications in a small fraction of the population[6]. An effective antiviral agent to treat all of these viruses would be valuable.

Historically, marboran was used to treat smallpox virus infections; however that drug was later found to be ineffective. The nucleoside analog ribavirin, in concert with immunoglobulin was effectively used to treat a vaccinia virus infection in an immunocompromisedindividual[6]. Nonetheless, currently there are no approved medications for smallpox or monkeypox infection. However, cidofovir (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine; HPMPC), a drug now in clinical use for treating severe herpesvirus infections also inhibits orthopoxviruses. Cidofovir, which is a derivative of cytosine, is approved for the treatment of cytomegalovirus (CMV) infection in immunocompromised patients, also inhibits the replication of vaccinia virus[7]. Cidofovir has a broad spectrum of activity against DNA viruses. The drug is protective in murine models of vaccinia virus and cowpox virus[7-10]. In in vitro models cidofovir is highly active against other orthopoxviruses: cowpox virus, camelpox, monkeypox, and 3 isolates of variola[10]. Cidofovir is being considered for licensure to treat smallpox, although renal failure is one of the frequent complications of treatment with the drug[11].

We present data showing that gemcitabine is much more potent than cidofovir at reducing replication of orthopoxviruses vaccinia and cowpox in vitro. These two viruses are used as surrogates to predict antiviral efficacy against the more important target variola or smallpox virus.

In the aftermath of the events of September and October 2001, there is concern that the variola virus might be used as an agent of bioterrorism. Since the events of September 11, 2001, and the increased risk of bio-terrorism there is a need for the

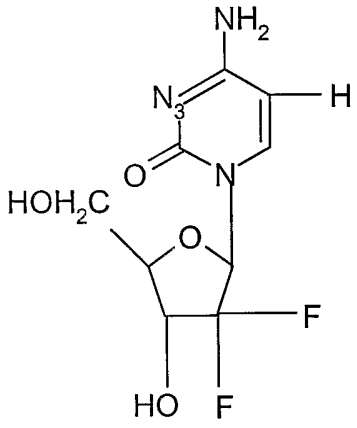
treatment of various viral scourges, specifically, small pox. No known treatment for a small pox infection has been heretofore discovered. We have discovered that gemcitabine would be an effective treatment for the disease caused by the smallpox virus.

Literature Cited

1. Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. Smallpox and its eradication. Geneva, Switzerland: World Health Organization; 1988.
2. Henderson DA. Bioterrorism as a public health threat. Emerg. Infect. Dis. 1998;4:488-92.
3. Breman J, Henderson DA. Poxvirus dilemmasmonkeypox, smallpox and biological terrorism. N. Engl. J. Med. 1998;339:556-9.
4. Russia, Iraq, and other potential sources of anthrax, smallpox, and other bioterrorist weapons. Committee on International Relations. First session, Washington, DC: United States Congress; 2001. p. 40.
5. Munoz-Perez MA, Rodriguez-Pichardo A, Camacho F, Colmenero MA. Dermatological findings correlated with CD4 lymphocyte counts in a prospective 3 year study of 1161 patients with human immunodeficiency virus disease predominantly acquired through intravenous drug abuse. Br. J. Dermatol. 1998;139:33-39.
6. Kesson AM, Ferguson JK, Rawlinson WD, Cunningham AL. Progressive vaccinia treated with ribavirin and vaccinia immune globulin. lin. Infect. Dis. 1997;25:911-14.
7. Naesens L, Snoeck R, Andrei G, Balzarini J, Neyts J, De Clercq E. HPMPC (cidofovir), PMEA (adefovir) and related acyclic nucleoside phosphonate analogues: a review of their pharmacology and clinical potential in the treatment of viral infections. Antivir. Chem. Chemother. 1997;8:1-23.
8. De Clercq E. Cidofovir in the treatment of poxvirus infections. Antiviral Research 2002;55(1):1-13.
9. Smee D, Sidwell R, Kefauver D, Bray M, Huggins J. Characterization of wild-type and cidofovir-resistant strains of camelpox, cowpox, monkeypox, and vaccinia viruses. Antimicrob Agents Chemother 2002;46(5):1329-35.

10. Bray M, Martinez M, Smee D, Kefauver D, Thompson E, Huggins J. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J Infect Dis 2000;181(1):10-9.

The present invention provides the treatment of smallpox and other poxvirus infections with a compound of the formula:



FORMULA I

which is 1-(4-amino-2-oxo-1H-pyrimidin-1-yl)-2,2-difluororibose, namely gemcitabine, also known as Gemzar®.

At present, with the increased risk of bio-terrorism after the terrible events of September 11, 2001, there is a need for the treatment of various viral scourges, like small pox. Gemcitabine, with its proven antiviral activity, would be an effective treatment for the disease caused by the smallpox virus.

Example 1

1-(2-oxo-4-amino-1H-pyrimidin-1-yl)-2-desoxy-2.2-difluororibose

Under nitrogen, to a solution of 47.3 g. of 3,5-bis(t-butyldimethylsilyloxy)-1-methanesulfonyloxy-2-desoxy-2,2-difluororibose in 940 ml. of methylene chloride was added 48 g. of bis(trimethylsilyl)-N-acetylcytosine. To this mixture was added 39.23 g. of trifluorome-thanesulfonyloxytrimethylsilane and the resulting mixture was cooled to room

temperature and 16 ml. of methanol was added thereto. The resulting solution was stirred for approximately 30 minutes and concentrated to about one-half of its original volume. The solution was cooled in ice and the precipitated solid was collected by filtration. The filtrate was shaken one time with approximately 300 ml. of 10% sodium bicarbonate and one time with 100 ml. of brine. The organic layer was evaporated to dryness in vacuo at 45° and the residue was dissolved in 1.3 1. of methanol saturated with ammonia. The resulting suspension was allowed to stir overnight at room temperature and the volatiles were removed under vacuum at 45°. The residue was dissolved in 275 ml. of methanol and 100 g. of Bio Rad ion exchange resin (AG 50WX8) was added thereto. The suspension was stirred at room temperature overnight and the resin was collected by filtration. The resin was rinsed with 100 ml. of methanol and suspended in 100 ml. methanol and 50 ml. of concentrated ammonium hydroxide. The resin containing suspension was stirred vigorously for 15 minutes and the resin was collected by filtration. This procedure was twice repeated with additional ammonia saturated methanol. The basic methanolic filtrates were combined and evaporated at 45° under vacuum to provide 13.8 g. of a solid. This material was chromatographed on a Waters Prep 500 C^{18} Reverse Phase Column with water as the eluent to provide 1.26 g. of 1-(2-oxo-4-amino-1H-pyrimidin-1-yl)-2-desoxy-2,2-difluororibose. nmr (CD_3OD, 90 MH_z) δ 3.7-4.65 (m, 4H), 4.8 (s, 4H), 5.97 (d, J=8 Hz, 1H), 6.24 (t, J=7 Hz, 1H), 7.88 (d, J=8 Hz, 1H); mass spec. m/e=263 =p.

The compound of the present method are preferably administered as a pharmaceutical formulation. Therefore, as yet another embodiment of the present invention, a pharmaceutical formulation useful for treating various diseases including smallpox in mammals is provided comprising a compound of formula I with a pharmaceutical carrier, diluent or excipient therefor.

The compounds are effectively administered orally, topically or parenterally. In general, dosage rates in the range of from about 5 mg./kg. to about 500 mg./kg. are useful. It is more preferred to administer rates in the range of from about 10 mg./g. to about 100 mg./kg.

Methods

In vitro Results:

Gemcitabine has not been tested against variola virus (smallpox) in either in vitro or in vivo systems. Those experiments must be done under biological safety level 4 conditions at the United States Centers for Disease Control or at the Russian State Research Center for Virology and Biotechnology at NPO Vector Koltsovo, Novosibirsk Region, Russia. Gemcitabine has been tested against three other members of the orthopox virus taxon, of which variola virus is a member. The other orthopox viruses are vaccinia virus, cowpox virus, and monkeypox virus. The capacity of gemcitabine to inhibit vaccinia virus was tested both at Eli Lilly and Company (Lilly) and at the University of Alabama at Birmingham (UAB). The capacity of gemcitabine to inhibit cowpox virus was tested both at the Pathology Division of the US Army Medical Research Institute of Infectious Diseases at Fort Detrick Maryland (USAMRIID) and at UAB. The capacity of gemcitabine to inhibit monkeypox virus was tested at USAMRIID.

For both the in vitro studies and in vivo studies, the drug cidofovir is included as a positive control for antiviral efficacy[10].

Protocols used for the in vitro experiments performed at Lilly

Virus pool preparation. Stock pools of the WR strain of vaccinia virus, which was originally obtained from the American Type Culture Collection, were grown in HeLa cells

Plaque reduction assay for efficacy. Confluent CV1 cells plated on six-well plates are inoculated with ~100 plaque forming units (pfu) of vaccinia virus, and incubated at 37°C with 5% CO_2 and 90% humidity. Nutrient agar-gemcitabine solutions are made containing various concentrations of gemcitabine (1-1,000 nM). After removing the vaccinia virus inoculum, the infected cell monolayers were overlaid with 3 ml of the 1% nutrient agar-gemcitabine solutions. The overlay solutions are allowed to solidify before the plates are incubated at 37°C with 5% CO_2 and 90% humidity for 3 days. Samples are tested in duplicate. Plaques are visualized by staining viable cells with neutral red. A 1 ml overlay of 1% agar and 0.025% neutral red is added on top of the original media. After a 5- to 6-hour incubation in the stain period the plaques are counted.

Results for the in vitro experiments performed at Lilly

Gemcitabine reduced vaccinia virus replication in a plaque reduction assay with an EC_{50} of less than 32 nM.

Table 1. Gemcitabine effect on vaccinia virus grown in CV1 cells.

Gemcitabine (nM)	Number of plaques
1000	0
500	0
250	0
125	0
62.5	0
31.3	14
15.6	21
7.8	25
3.9	22
2.0	25
1.0	23
Control	21

Protocols used for the in vitro experiments performed at UAB[14]

Virus pool preparation. Stock pools of the Copenhagen strain of vaccinia virus strain and the Brighton strain cowpox virus were obtained from John Huggins of the U.S. Army Medical Research Institute for Infectious Diseases, Frederick, Md. These pools were prepared in Vero cells. At Earl Kern's laboratory at the University of Alabama at Birmingham the virus pools are diluted 1:50 to make working stocks.

Plaque reduction assay for efficacy. Human foreskin fibroblast cells are plated on six-well plates two days prior to use, and incubated at 37°C with 10% CO_2 and 90% humidity. Gemcitabine solutions are made at twice the desired concentration in 2x minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and antibiotics and diluted serially 1:5 in 2x MEM to provide six concentrations of drug. Poxvirus stocks are diluted in MEM containing 10% FBS to a desired concentration giving 20 to 30 plaques per well. To perform the plaque reduction assay the medium is

aspirated from the wells, and 0.2 ml of virus is added to each well. Samples are tested in triplicate. To measure gemcitabine toxicity 0.2 ml of medium is added to drug toxicity wells that do not receive virus. To allow the virus to adsorb, the plates are incubated for 1 hour, during which they are shaken every 15 min. After the virus adsorption period, the 2X media-gemcitabine mixture are mixed with equal volumes of 1% agarose. This yielded final gemcitabine concentrations with a maximum amount of 100 μM and a final agarose overlay concentration of 0.5%. Two ml of the gemcitabine-agarose mixture was added to each well, and the plates are incubated for 3 days. The plates are then assayed by staining the cells with a 1.5% solution of neutral red. After a 5- to 6-h incubation in the stain period, the solution is aspirated and the plaques are counted using a stereomicroscope at 10X magnification. The 50% effective concentrations (EC_{50}) are calculated using the computer program MacSynergy II, version 1.

Neutral-red uptake assay for toxicity. Human foreskin fibroblast cells are plated in 96-well plates twenty-four hours prior to the assay at a concentration of 2.5×10^5 per ml. After a 24-hour incubation at 37° in 5% CO_2, the medium is removed and 125 μl of gemcitabine is added to the first row of wells. The medium containing the gemcitabine is then diluted serially 1:5. After gemcitabine addition, the plates were incubated in CO_2 incubator at 37°C. After a 7 day incubation, the gemcitabine-containing medium is removed, and 200 μl of 0.01% neutral red in phosphate-buffered saline (PBS)/well is added and incubated for 60 minutes. The dye solution is removed, and the cells are washed with PBS. The PBS wash is removed, and 200 μl of 50% ethanol-1% glacial acetic acid (in H_2O)/well is added to each well. The plates were placed on a rotary shaker for 15 min, and the optical densities were read at 540 nm. The CC_{50} is calculated using the MacSynergy II version1.

Results for the in vitro experiments performed at UAB

In both the cowpox virus and vaccinia virus assays gemcitabine has potent antiviral activity (Tables 2 and 3). The activity in the plaque reduction assays is significantly greater than in CPE inhibition assays; however this is also true for cidofovir. When compared on a molar basis to cidofovir, gemcitabine is much more active at lease at the EC_{50} level for CPE inhibition, and at the EC_{50} and EC_{90} levels for plaque reduction in both vaccinia and cowpox viruses. In the neutral red uptake assay for cytotoxicity in

stationary human foreskin fibroblasts cells gemcitabine has a CC_{50} of > 10 μg/ml (3.3 μM). In that assay cidofovir has a CC_{50} of > 100 μg/ml (358 μM).

Table 2. Gemcitabine and cidofovir effects on vaccinia virus grown in human foreskin fibroblast cells.

Assay	EC_{50}	EC_{90}	CC_{50}	SI
Gemcitabine CPE Inhibition	0.09 μg/ml (0.30 μM)	10 μg/ml (3.3 μM)	>10 μg/ml (3.3 μM)	>114
Gemcitabine Plaque Reduction	0.004 μg/ml (0.013 μM)	0.008 μg/ml (0.027 μM)	>1 μg/ml (0.33 μM)	>250
Cidofovir CPE Inhibition	2 μg/ml (7.2 μM)	3.2 μg/ml (11.5 μM)		
Cidofovir Plaque Reduction	11.7 μg/ml (41.9 μM)	20.8 μg/ml (74.5 μM)		

Table 3. Gemcitabine and cidofovir effects on cowpox virus grown in human foreskin fibroblast cells.

Assay	EC_{50}	EC_{90}	CC_{50}	SI
Gemcitabine CPE Inhibition	0.01 μg/ml 0.02 (0.033 μM)	10 μg/ml (3.3 μM)	>10 μg/ml (3.3 μM)	>1000
Gemcitabine Plaque Reduction	0.005 μg/ml (0.017 μM)	0.026 μg/ml (0.087 μM)	>1 μg/ml (0.33 μM)	>250
Cidofovir CPE Inhibition	2.2 μg/ml (7.9 μM)	3.9 μg/ml (14.0 μM)		
Cidofovir Plaque Reduction	11.0 μg/ml (39.4 μM)	47.9 μg/ml (172 μM)		

Protocols used for the in vitro experiments performed at USAMRIID

Virus pool preparation. Stock pools of the Copenhagen strain of vaccinia virus strain and the Zaire strain monkeypox virus were obtained from Joseph Esposito of the U.S. Centers for Disease Control and Prevention, Atlanta GA. These pools were prepared in African green monkey kidney (Vero 76) cells.

Plaque reduction assay for efficacy. The assay is described in detail in Smee et al. and outlined below (Smee D, Bray M, Huggins J. Antiviral activity and mode of action studies of ribavirin and mycophenolic acid against orthopoxviruses in vitro. Antivir Chem Chemother 2001;12(6):327-35). Vero 76 cells are plated on six-well plates two days prior to use, and incubated at 37°C with 10% CO_2 and 90% humidity. Once the cells form confluent monolayers, plates are infected with about 100 plaque forming units (pfu) of

virus per well. The virus is adsorbed for 1.5-2 hours, and then three fold dilutions of gemcitabine were applied. The maximum gemcitabine concentration tested was 100 μg/ml. (33 μM). Monkeypox infected plates are then incubated at 37°C with 10% CO_2 and 90% humidity for 6 days, and cowpox infected plates for 4 days. At this point plaque diameters for both viruses are about 3 mm. Cells are fixed and stained in 3% buffered formalin/0.1% crystal violet for 15 minutes. Plaques are then counted. The concentration of gemcitabine that reduces virus plaque numbers by 50% (EC_{50}) is determined by plotting the drug concentration versus the ratio of number of plaques in drug treated samples relative to control samples on a semi log scale. Cytotoxicity was measured using a neutral red uptake assay as described in Smee et al..

Results for the in vitro experiments performed at USAMRIID

In plaque reduction assays gemcitabine does not reduce monkeypox titers at or below concentrations of 100 μg/ml. (33 μM). In the cowpox plaque reduction assay gemcitabine was highly active with an EC_{50} of 0.005 μg/ml (0.017 μM). This compares to an EC_{50} value for cidofovir against cowpox in this assay of 92±4 μM (Smee et al.). Thus on a molar basis gemcitabine is more than 5000 more active against cowpox than cidofovir, the drug currently planned for use to treat smallpox infected patients. In neutral red uptake assays, no gemcitabine-induced toxicity was evident at 100 μg/ml for on confluent Vero 76 cells.

In vivo experiments performed at UAB and USAMRIID:

Although there are no murine models for variola virus infections, murine models have been reported for cowpox and vaccinia virus infections. In those models the nucleoside analog cidofovir has proved to be an effective anti-orthopoxvirus agent [8, 10]. In fact the validity of those murine models for identifying anti-orthopoxvirus agents is largely based on the results obtained using cidofovir, which is an acyclic nucleoside phosphonate whose antiviral mechanism of action is through termination of DNA synthesis[16]. Experiments in which BALB/c mice were infected intranasally with cowpox and then treated using a single or multiple doses of intraperitoneally injected gemcitabine failed to demonstrate any in vivo efficacy. In three different experiments (Tables 5 & 6 show work done at UAB, Table 7 shows work done at USAMRIID), the mean number of days until death as

a result of the cowpox infection was not significantly greater in the gemcitabine treated mice relative to the placebo treated or control mice. All cidofovir treated mice survived the cowpox infection. Furthermore, at the higher doses tested a number of mice died. No gemcitabine-induced mortality was noted at the gemcitabine doses previously shown by Lilly to be tolerated by mice. The vaccinia virus model experiment, (Table 4, showing work done at UAB) showed no mouse mortality as a result of the intranasal vaccinia infection, so those data do not address gemcitabine in vivo efficacy.

Table 4. Effect of intraperitoneal treatment with gemcitabine and cidofovir on the mortality of BALB/c mice inoculated intranasally with Vaccinia, WR

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
Placebo Saline	0/15	0	---	---	---
Cidofovir 10 mg/kg +24 hr	1/15	7	---	14.0	---
Gemcitabine 10 mg/kg +24 hr	15/15	100	---	5.9	---
3 mg/kg +24 hr	15/15	100	---	6.5	---
1 mg/kg +24 hr	6/15	40	---	9.3	---
	Toxicity				
Gemcitabine 10 mg/kg	10/10	100	---	5.9	---
3 mg/kg	9/10	90	---	6.1	---
1 mg/kg	3/10	30	---	7.7	---

a Gemcitabine and cidofovir were prepared in sterile saline and delivered intraperitoneally in 0.1 ml doses. Animals were treated twice daily for 5 days beginning 24 hours post viral inoculation. With the gemcitabine dose groups, the 10 mg/kg and 3 mg/kg groups were discontinued after 4 days dosing due to toxicity

MDD = Mean Day of Death

Table 5: Effect of intraperitoneal treatment with gemcitabine and cidofovir on the mortality of BALB/c mice inoculated intranasally with Cowpox, Brighton.

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
Placebo Saline	10/15	67	---	12.0	---
Cidofovir 10 mg/kg +24 hr	0/15	0	<0.001	---	<0.001
Gemcitabine 10 mg/kg +24 hr	15/15	100	<0.05	6.0	<0.001
3 mg/kg +24 hr	15/15	100	<0.05	6.8	<0.001
1 mg/kg +24 hr	15/15	100	<0.05	10.03	<0.001
	Toxicity				
Gemcitabine 10 mg/kg	10/10	100	0.06	5.9	---
3 mg/kg	9/10	90	NS[c]	6.1	---
1 mg/kg	3/10	30	NS	7.7	---

a Gemcitabine and cidofovir were prepared in sterile saline and delivered intraperitoneally in 0.1 ml doses. Animals were treated twice daily for 5 days beginning 24 hours post viral inoculation. With the gemcitabine dose groups, the 10 mg/kg and 3 mg/kg groups were discontinued after 4 days dosing due to toxicity

b. MDD = Mean Day of Death

c. NS = Not significant when compared to the placebo control

Table 6. Effect of intraperitoneal treatment with gemcitabine and cidofovir on the mortality of BALB/c mice inoculated intranasally with Cowpox, Brighton.

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
Placebo Saline	15/15	67	---	8.6	---
Gemcitabine 1.0 mg/kg +24 hr	15/15	100	---	9.3	0.01
0.3 mg/kg +24 hr	15/15	100	---	8.9	NS
0.1 mg/kg +24 hr	15/15	100	NS[c]	9.6	0.05
0.03 mg/kg +24 hr	15/15	100	---	9.1	NS
Toxicity					
Gemcitabine	0/10	0	<0.001	---	---

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
1.0 mg/kg					
0.3 mg/kg	0/10	0	<0.001	---	---
0.1 mg/kg	0/10	0	<0.001	---	---
0.03 mg/kg	0/10	0	<0.001	---	---

a Gemcitabine and cidofovir were prepared in sterile saline and delivered intraperitoneally in 0.1 ml doses. Animals were treated once daily for 5 days beginning 24 hours post viral inoculation

b. MDD = Mean Day of Death

c. NS = Not significant when compared to the placebo control

Table 7. Effect of intraperitoneal treatment with gemcitabine and cidofovir on the mortality of BALB/c mice inoculated intranasally with Cowpox, Brighton.

Treatment[a]	**Mortality Number**	**Percent**	**MDD[b]**
Control	10/10	100	8.2
Gemcitabine 40 mg/kg QD 0,6	10/10	100	8.0
10 mg/kg QD 0,3,6,9	10/10	100	7.8
5 mg/kg QD 0,3,6,9	10/10	100	8.3
1 mg/kg QD X 5	10/10	100	8.5
Placebo 1 mg/kg QD X 5	10/10	100	7.3
Cidofovir 100 mg/kg once	0/10	0	---
Toxicity			
Gemcitabine 40 mg/kg QD 0,6	0/5	0	---
10 mg/kg QD 0,3,6,9	0/5	0	---
5 mg/kg QD 0,3,6,9	0/5	0	---
1 mg/kg QD X 5	0/5	0	---
Placebo 1 mg/kg QD X 5	0/5	0	---
Cidofovir 100 mg/kg once	0/5	0	---

a Gemcitabine and cidofovir were prepared in sterile saline and delivered intraperitoneally in 0.1 ml doses. Animals were treated as noted beginning 24 hours post viral inoculation

b. MDD = Mean Day of Death

5

WE CLAIM:

1. A method of treating smallpox in a mammalian patient in need thereof comprising administering a therapeutically effective dose of the compound 1-(4-amino-2-oxo-1H-pyrimidin-1-yl)-2,2-difluororibose, namely gemcitabine, to the patient.