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The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies

B.G. Murphy[a], M. Perron[c], E. Murakami[c], K. Bauer[a], Y. Park[c], C. Eckstrand[a], M. Liepnieks[a], N.C. Pedersen[b,*]

[a] Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, CA, USA
[b] Center for Companion Animal Health, School of Veterinary Medicine, University of California, One Shields Ave., Davis, CA, USA
[c] Gilead Sciences, Foster City, CA, USA

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ABSTRACT

Feline infectious peritonitis (FIP) is a common and highly lethal coronavirus disease of domestic cats. Recent studies of diseases caused by several RNA viruses in people and other species indicate that antiviral therapy may be effective against FIP in cats. The small molecule nucleoside analog GS-441524 is a molecular precursor to a pharmacologically active nucleoside triphosphate molecule. These analogs act as an alternative substrate and RNA-chain terminator of viral RNA dependent RNA polymerase. We determined that GS-441524 was non-toxic in feline cells at concentrations as high as 100 uM and effectively inhibited FIPV replication in cultured CRFK cells and in naturally infected feline peritoneal macrophages at concentrations as low as 1 uM. We determined the pharmacokinetics of GS-441524 in cats *in vivo* and established a dosage that would sustain effective blood levels for 24 h. In an experimental FIPV infection of cats, GS-441524 treatment caused a rapid reversal of disease signs and return to normality with as little as two weeks of treatment in 10/10 cats and with no apparent toxicity.

1. Introduction

Feline infectious peritonitis (FIP) is a well-documented infectious disease of cats, especially kittens, adolescents and young adults from shelters, kitten foster/rescues and catteries (Pedersen, 2014b). The causative FIP virus (FIPV) is a positive single stranded RNA virus belonging to the family *Coronaviridae,* species Alphacoronavirus 1, subspecies feline coronavirus, biotype FIP virus (FIPV) (Tekes and Thiel, 2016).

FIPV infection has a complex immunopathogenesis, a feature shared by other coronavirus infections (de Wilde et al., 2017), and has become one of the most researched infectious diseases of cats since its discovery over a half century ago (Kipar and Meli, 2014; Pedersen, 2014a, 2014b; Tekes and Thiel, 2016). Despite these advances, modern treatment options for FIP remain palliative and vaccines have proven either unsafe or ineffective (Pedersen, 2014b). The emergence of SARS-associated coronavirus has provided an impetus for an investment in antiviral discovery focused on coronaviruses (De Clercq, 2004). A 3C-like protease inhibitor (GC376) was first demonstrated to be highly effective against experimental FIPV infections (Kim et al., 2016). A subsequent study on cats with naturally acquired FIP showed them to be much more difficult to treat (Pedersen et al., 2017). Nevertheless, this was the first study demonstrating the potential for an antiviral compound to treat FIP in nature and 6 of 20 cats have remained disease free for well over one year after 12 weeks of treatment (Pedersen et al., 2017).

GS-441524, a 1′-cyano-substituted adenine C-nucleoside ribose analogue (Fig. 1A), is a small molecule that exhibits potent antiviral activity against a number of RNA viruses, including the zoonotic severe acute respiratory syndrome (SARS) coronavirus (Cho et al., 2012). A phosphoramidate prodrug of GS-441524 (GS-5734) has been previously shown to inhibit the replication of several taxonomically diverse RNA viruses such as Middle East respiratory syndrome virus, Ebola virus, Lassa fever virus, Junin virus and respiratory syncytial virus, while having low cytotoxicity in a wide-range of cell lines (Sheahan et al., 2017). GS-5734 has also been shown to protect rhesus monkeys from experimental Ebola virus infection (Warren et al., 2016).

GS-441524 requires intracellular phosphorylation via cellular kinases to a nucleoside monophosphate and subsequently to the active triphosphate metabolite (NTP) (Cho et al., 2012; Sheahan et al., 2017; Warren et al., 2016) (Fig. 1A). The active NTP analog functions as a competitor of the natural nucleoside triphosphates in viral RNA synthesis. The active form of GS-441524 has been shown to inhibit RSV

* Corresponding author at: Center for Companion Animal Health, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA, 95616, USA.
E-mail address: ncpedersen@ucdavis.edu (N.C. Pedersen).

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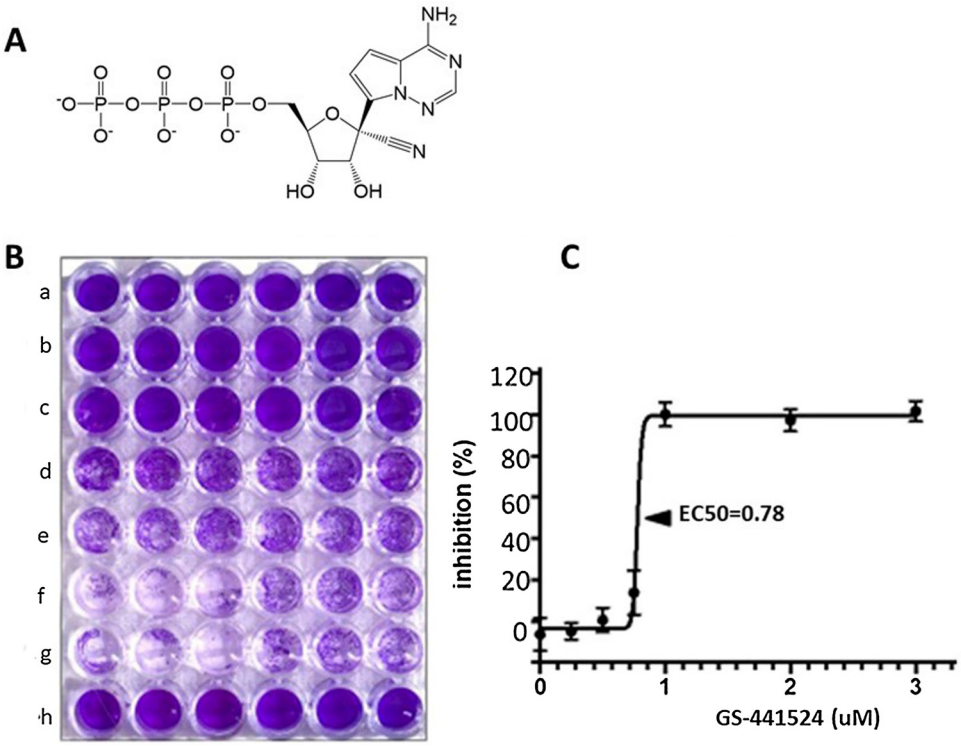

Fig. 1. A. Molecular structure of triphosphorylated GS-441524. B. CRFK cell cultures infected with FIPV-79-1146 showing typical CPE and crystal violet staining pattern. Infected cells were treated with 3 uM (row a), 2 uM (row b), 1 uM (row c), 0.75 uM (row d), 0.5 uM (row e), 0.25 uM (row f), 0 uM GS-441524 (row g); untreated and uninfected cells (row h). C. A nonlinear regression analysis utilizing these data determined the EC50 for GS-441524 to be 0.78 uM.

RNA-dependent RNA polymerase mediated transcription by incorporating into the nascent viral transcript and causing premature termination (Sheahan et al., 2017). We hypothesized that GS-441524 would be activated in feline cells, attenuate FIPV replication, have low cytotoxicity in feline cells *in vitro* and effectively treat cats with experimentally induced FIP.

2. Materials and methods

2.1. Experimental animals

Specific pathogen free (SPF) cats were purposefully bred in the Feline Research Laboratory (FRL) breeding colony of the Feline Nutrition Center, UC Davis. Twelve adolescent cats were used for experimental infections and six young adult cats for pharmacokinetic studies. Cats on experiment were housed in open rooms in facilities of the FRL and cared for by the FRL staff of animal caretakers. Experiments involving the use of laboratory bred cats were conducted under UC Davis IACUC protocol #19936. The UC Davis Policy on the Care and Use of Animals in Teaching and Research requires that University practices for the procurement, housing, and care and use of animals must conform to: 1) the ILAR *Guide for the Care and Use of Laboratory Animals*; 2) the *Guide for the Care and Use of Agricultural Animals in Research and Teaching*; 3) all regulations of the United States Department of Agriculture (USDA) issued by the USDA implementing the Animal Welfare Act (AWA) and its amendments (9 CFR, Chapter 3); and 4) the Public Health Service's *Policy on Humane Care and Use of Laboratory Animals*. In addition, University policy requires that all facilities in which animals are housed, and the programs associated with those facilities, must be accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International.

2.2. FIPV-79-1146 inoculum for in vitro CRFK infection studies

Crandell-Rees feline kidney (CRFK) cells were propagated in 250 ml flasks with 25 ml DMEM/10%FBS and infected at 70% confluency with 1 ml cell-culture supernatant containing 5×10^5 tissue culture infectious doses-50% (TCID50) per ml of serotype II FIPV WSU-79-1146 (GenBank DQ010921) and incubated for 48 h. Flasks were frozen at −70 °C for 8 min and the thawed mixture of cells and original culture fluid centrifuged to remove cellular and subcellular debris and supernatant stored in liquid nitrogen. The level of infectious virus in an aliquot of the culture supernatant was then determined. CRFK cells were grown as described above in 24 well plates and infected with one ml/well of a serial 10-fold dilution of the frozen supernatant. Plates were then incubated for 48 h, stained with crystal violet (Fig. 1B), and each well was scored for CPE. A TCID50 was calculated from six replicates by the Reed-Muench method.

2.3. Quantitation of FIPV replication by qRT-PCR

Viral RNA was isolated from both the culture supernatant (QIAamp Viral RNA Mini Kit (Qiagen) and pelleted cells (RNAeasy, Qiagen) according to the manufacturer's instructions. DNase treatment of isolated RNA was accomplished with TURBO DNase (Ambion). RNA was reverse transcribed into cDNA with the Origene First-Strand cDNA Synthesis System for qRT-PCR (Origene). A control reaction excluding reverse transcriptase was included for each sample. FIPV RNA copy numbers were measured by qRT-PCR as previously described (Murphy et al., 2012). PCR primers were based on a consensus sequence of the feline coronavirus 7b gene. The sequence for the forward primer was 5′-GGA AGT TTA GAT TTG ATT TGG CAA TGC TAG and the sequence for the reverse primer 5′-AAC AAT CAC TAG ATC CAG ACG TTA GCT. A control reaction excluding cDNA (water template) was included for each assay. Real-time PCR for the GAPDH housekeeping gene (GAPDH) was performed in parallel and results were normalized per 10^6 copies of GAPDH (13). Quantification of FIPV RNA copy number was based on a standard curve generated from viral transcripts prepared by *in vitro* transcription of a plasmid (pCR2.1, Invitrogen) containing a 112 nucleotide-long amplicon.

2.4. GS-441524

GS-441524 was provided by Gilead Sciences, Inc.