Title

Are unlicensed feline coronavirus antiviral compounds GS-441524 and GC376 what they claim to be? A qualitative and quantitative analysis.

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Author Contributions

Conceptualization, M.E.B.; methodology, M.E.B.; validation, A.J.M.; formal analysis, A.J.M. and M.E.B.; investigation, A.J.M.; resources, M.E.B.; data curation, A.J.M. and M.E.B.; Visualization, A.J.M. and M.E.B.; writing—original draft preparation, M.E.B.; writing—review and editing, A.J.M. and M.E.B.; supervision, M.E.B.; project administration, M.E.B.; funding acquisition, M.E.B. and A.J.M. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Abstract

Background

Feline Infectious peritonitis (FIP) is a disease caused by feline coronavirus. FIP is 100% fatal and was considered untreatable until recently. Although there is no FDA-approved treatment for FIP, experimental drugs, GS-441524 (the primary metabolite of remdesivir), GC376 (a viral protease inhibitor), and Molnupiravir have been found effective in treating this otherwise fatal disease. Because of the severity of FIP and lack of approved treatments, these drugs are sometimes used off-label to treat this disease. These drugs can be obtained through crowdsource online social media groups.

Methods

We tested 28 vials from 10 brands of GS-441524 and 5 vials from one brand of GC376. We compared the GS-441524 to a control standard from Ambeed and the GC376 to a standard from Cayman Chemical. We recorded physical appearance, pH, absorbance, high-performance liquid chromatography retention times, and thin-layer chromatography retention factors for all of them. Some were also used for nuclear magnetic resonance and mass spectrometric analysis.

Objectives

These medications are unregulated, and therefore come with potential risks for animals being treated. This study set out to determine the purity of various batches of these drugs from several companies, characterizing them, and noting any impurities or other

unusual characteristics. We also developed a method to qualitatively assess the primary components prior to administration.

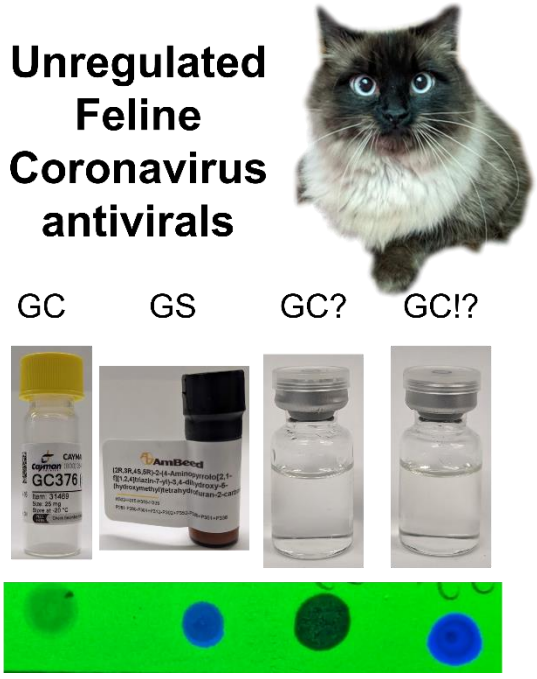
Results

Most of the GS-441524 samples tested were similar in purity and composition, both between batches and between brands. Most of the brands tested had concentrations of GS-41524 similar to the concentration advertised. 3 of the 17 types of vials tested had concentrations over 10% above advertised, and one was over 20% higher than advertised. One batch, known to have significant quality concerns based on the lack of efficacy in animals treated, had a concentration almost 50% below advertised. We also tested 5 vials of GC376 and found 1/5 vials contained GS-441524 rather than GC376 and the other 4/5 vials contained Molnupiravir.

Conclusions

Most of the vials tested contained the advertised drug at the labeled concentration. Some of the GS-441524 vials were 10-25% more concentrated than advertised but given the low toxicity this is probably not of great concern. However, one batch had approximately half the expected concentration, which explains the reported lack of efficacy. None of the GC376 vials tested contained GC376. GC376 is used in cats that are unresponsive to GS-441524, and use of the wrong antiviral can cause serious side effects. In addition, we provide suggested methods for determining the purity of new batches of these compounds and distinguishing one drug from the other.

Graphical Abstract



Feline infectious peritonitis (FIP) caused by feline coronavirus was, until recently, considered both fatal and untreatable. Experimental antivirals such as GS-441524 and GC376 can be obtained off-label through crowdsourced internet groups and have been used by pet owners to treat FIP. We describe analysis and characterization of unregulated batches of both GS-441524 and GC376.

Keywords

Feline coronavirus, Feline infectious peritonitis, GS-441524, GC376, Off-label Use, Molnupiravir

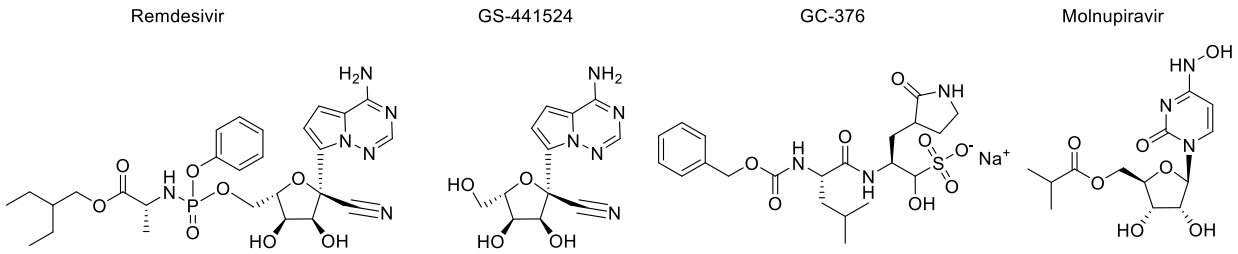
Main text

Introduction

Coronaviruses have long been circulating in human and animal populations [1]. Until recently, 15-30% of common colds in humans were caused by coronaviruses and were generally not considered life-threatening [2]. The COVID-19 pandemic fueled by the SARS-CoV-2 virus has heightened our appreciation for the destructive power of coronaviruses [3]. While reports of domestic, caged, and wild felines being infected with the SARS-CoV-2 virus were widely reported,[4], a feline coronavirus (FCoV) has long been circulating among felines in captivity and in the wild [1,5–7].

Feline coronavirus (FCoV) starts in cats as a gastrointestinal disease known as feline enteric coronavirus (FECV). It has been reported that 88% of the time, the affected cats experience a mild bout of diarrhea and recover fully, but in 12% of cats, more commonly in kittens and immunocompromised cats, FECV mutates within the individual to feline infectious peritonitis virus (FIPV). The precise sequence changes required to cause the FECV to FIPV transition are thought to involve one or more genetic changes to the viral 3c gene and/or changes to the viral S protein which allow FIPV to infect not only the gastrointestinal tract, but also macrophages [8–12]. This triggers an intense inflammatory reaction resulting in swelling of blood vessels which develops into feline infectious peritonitis (FIP) [8,9,11,13]. Once a cat develops symptoms of FIP it is 100% fatal and until recently was considered incurable [11,14]. Recent advances in antiviral therapies led to the design of new, effective nucleoside antimetabolites [13]. One such

compound, GS-441524, in Scheme 1 showed efficacy against feline coronavirus and low toxicity in cat cells [13].



Scheme 1. Structures of, remdesivir, GS-441524, and GC376.

GS-441524 was first synthesized in 2009 [15], the compound was shown to be efficacious against FIP in 2018 [13], and preliminary results of the first feline clinical trials were reported in 2022 [16]. However, during the intervening years feline enthusiasts have sought a cure for pets fatally diagnosed with FIP. Small manufacturing companies saw a potential market and started producing unregulated GS-441524 [17]. Crowdsourced groups orchestrate the logistics of shipping and distribution of GS-441524 worldwide [18].

Occasionally, a cat presents with FIP that is not effectively treated with GS-441524, so these groups also offer other antivirals shown to be effective in treating FIP such as GC376 or Molnupiravir shown above in scheme 1[19,20]. It was discovered that GC376 in combination with GS-441524 shortened treatment duration compared to either drug alone.[21] GC376 binds to and inhibits the main protease of several RNA viruses including coronaviruses such as SARS, MERS, and FCoV but also other RNA viruses such as norovirus.[19,22,23] GC376 is a protease inhibitor and acts through a different mechanism than GS-441524.[24] RNA viruses like COVID-19 and FCoV transcribe and

translate their genes into long polyprotein chains which must be cleaved for effective reproduction, and GC376 inhibits the protease responsible for this cleavage. Molnupiravir acts on the RNA-dependent RNA polymerase similarly to Remdesivir and GS-441524.

The administrators of the crowdsource groups have reported that some vials of antivirals were more effective than others [personal communication]. Because it can cost up to $5000 for a successful course of these unlicensed medications, unproven batches of drug are provided at steep discounts to rescues and cat owners and the rate of recovery of cats treated with unproven batches is compared to proven batches to determine batch quality [25]. This method of testing is imprecise and can be dangerous for companion animals but is a calculated risk of using drugs without FDA approval and regulation. In this work we examine several batches of GS-441524 and GC376 obtained from these groups from different manufacturers. We tested for purity and concentration and characterized the components of these drugs.

Materials and Methods

Materials

MilliQ water was used throughout the experimental preparation and for dilutions. HPLC grade acetonitrile was obtained from Oakwood Chemical. Trifluoroacetic acid was purchased from Millipore Sigma. Western Family 70% isopropanol was obtained from Smith's pharmacy. DMSO-d6 was obtained from Cambridge Isotope Laboratories, Inc. Optima grade methanol for mass spectrometry samples was obtained from Fisher

Chemical. UV-Cuvettes were obtained from GMBH. GS-441524 samples were generously supplied by members of the crowdsourced FIP treatment groups with the exception of the “Panda” brand which was donated by a pet owner who sourced them from MaxPaw.

pH measurements

pH measurements were measured both using pH paper and a pH probe. For the GS-441524 vials which were larger, we used a Mettler Toledo LE410 probe with a Mettler Toledo five easy plus meter. The GC376 vials were smaller and were measured using an STmidro5 probe from Ohaus and a Starter 3100 meter from Ohaus.

High performance liquid chromatography

The HPLC system is an Agilent Series 1260 infinity II (Model #G7157A, Agilent Technologies, Palo Alto, CA, USA) with a variable wavelength detector (Model #G7114A, Agilent Technologies, Palo Alto, CA, USA) and detected using a 1260 infinity II fraction collector (Model #G1364E, Agilent Technologies, Palo Alto, CA, USA). All the analyses of data were done using Agilent OpenLab CDS ChemStation Edition Ver. Rev. C. 01.10[201]. The separation protocol was 45 minutes long, using an Xbridge prep C18 5 µM OBD column starting at 5% buffer B and going to 100% buffer B in the first 35 minutes, then staying at 100% B for 10 minutes. Buffer A was 99.9% MilliQ water with 0.1% trifluoroacetic acid, and buffer B was 90% acetonitrile, 9.3% MilliQ water, and 0.7% trifluoroacetic acid.

Thin layer chromatography

Thin layer chromatography (TLC) is described in detail in supplemental information appendix A. Briefly, TLC silica gel 60 F254 from Millipore was used. Samples were diluted 1:100 in water before application. The GS plates were developed in 100% isopropanol from Fisher Scientific, and the GC376 containing plates were developed in pure ethyl acetate.

Spectroscopy

Absorbance spectra and fluorescence were measured using a Molecular Devices SpectraMax M5 spectrophotometer and were recorded and analyzed using SoftMax Pro 7.1 from Molecular Devices. Samples were diluted 1:1000 in MilliQ water and pipetted into a disposable UV-cuvette semi-micro from GMBH. Absorbance was measured at 240 nm.

Mass Spectrometry

Mass spectrometry was performed at either the University of Utah Department of Chemistry Mass Spectrometry Facility with a Waters Xevo G2S Q-ToF w/Acquity I Class UPLC Tandem Mass Spec, or on a Waters Acquity QDA detector in house. Mass spectra are recorded in positive ion mode. In the mass spectrometry facility, MassLink Workstation software, including Qualitative Analysis (version B.07.00), was used for processing both raw MS and MS-MS data, including molecular feature extraction, background subtraction, data filtering, and molecular formula estimation. The raw data were processed using the Find by Molecular Feature (MF) algorithm called Molecular Feature Extractor (MFE) within MassHunter Qualitative Analysis software. Extracted

molecular features were processed to create a list of compounds. Data recorded on the QDA detector were analyzed using Mass Lynx V4.2.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectra were recorded at the University of Utah Nuclear Magnetic Resonance core. 1H and 13C NMR spectra were on a Varian Mercury 400 MHz spectrometer. 2D NMR spectra were recorded on a Varian Inova 500 MHz spectrometer. 1H-NMR spectra were acquired at 400 MHz and the chemical shifts (δ) of proton resonances were reported relative to the residual solvent peak (2.50 ppm for DMSO-d6).

Results

Samples

For GS-441524, we obtained samples with 17 different labels, 10 which are seen in Table 1. For Oscar, Panda, Shire, and Trusted we tested multiple vials. We measured absorbance and pH on 4 vials of Oscar, 8 vials of Panda, 8 vials of Shire and 2 vials of Trusted. In addition, we received and analyzed 5 vials of GC376 all from the same manufacturer, 金俊, jīn jùn, which we refer to as JJ. Photos and an inventory of the tested vials are shown in Figure S1.

Comparison of GS-441524 between manufacturers and vials

We measured the pH of each sample using both pH paper and a pH probe, and all of the vials measured similarly in low pH, all are under 2 in agreement with the original safety and efficacy study of GS-441524 which used a pH of 1.5 [26]. The low pH is necessary to solubilize and stabilize GS-441524 with the most common diluent conditions. We also ran TLC plates and calculated retention factors for most of the brands seen in table 1 and Figure S2.

We then ran absorbance spectra on all of the samples from 200 nm to 500 nm with 5 nm increments. The absorbance spectra of select samples are shown in Figure 1A. We found that most of the GS-441524 samples absorbed similarly, however one, Shire, was much lower than all the rest, so next we decided to calculate the concentrations of each GS-441524 vial.

We used the positive control purchased from Ambeed to make GS-441524 standard curves at 3 different concentrations shown in Figure S3. We determined that the

Brand	Advertised concentration	Calculated concentration	pH	220 nm Purity (%)	Retention time	Retention factor
Shire	15 mg/mL	8.73 mg/mL	1.31	84.9	6.281	0.5
Seka	15 mg/mL	14.5 mg/mL	1.99	90.0	6.334	0.5
Trusted	15 mg/mL	14.7 mg/mL	0.61	85.0	6.345	0.5
Lucky 15	15 mg/mL	16.0 mg/mL	1.60	92.8	6.402	0.5
Oscar	15 mg/mL	17.7 mg/mL	1.82	88.4	6.426	0.5
Valor	17 mg/mL	17.8 mg/mL	1.58	86.3	6.263	0.5
Panda	15 mg/mL	18.3 mg/mL	150	88.7	6.346	0.5
Rainbow	20 mg/mL	19.9 mg/mL	1.64	89.3	6.403	0.5
Ohana	20 mg/mL	19.9 mg/mL	1.53	91.9	6.364	0.5
Karma	18 mg/mL	20.1 mg/mL	1.62	88.5	6.318	0.5
Ambeed	n/a	n/a	n/a	91.9	6.297	0.5

Table 1. Results of testing of 10 brands of unregulated GS-441524 from lowest to highest calculated concentration and a positive control from Ambeed. Concentration was measured using absorbance at 240 nm. pH was measured using a pH probe. Retention time and purity were measured using high-performance liquid chromatography. The retention factors were calculated using TLC plates developed in pure isopropanol.

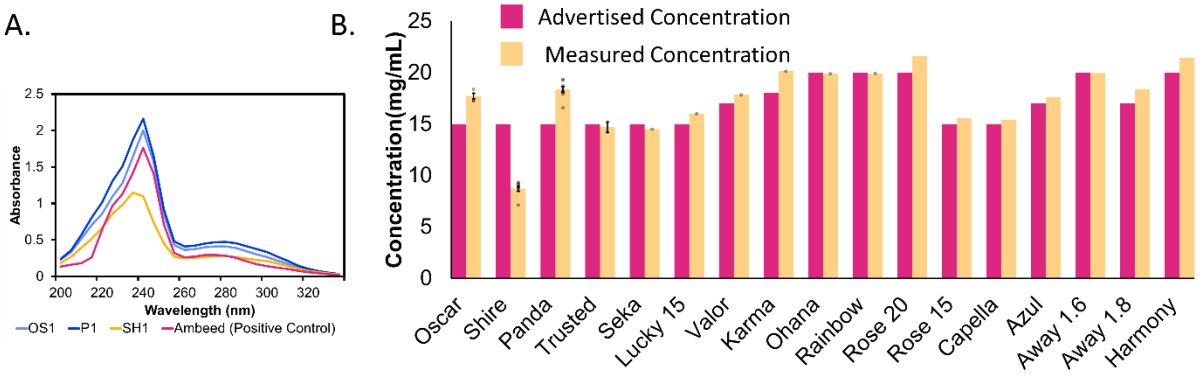


Figure 1. Comparison of brands of Gs-441524 A. Line graph showing the absorbance spectra of 3 brands and the GS-441524 positive control that are all advertised to be 15 mg/mL. Os1, P1 and Sh1 refer to Oscar vial 1, Panda vial 1, and Shire vial 1 respectively. B.. A bar graph showing the advertised concentrations all 17 GS-441524 brands tested in pink, and the measured concentration in yellow. When multiple vials were tested, the measured concentration for each individual vial is shown as a dot, the average concentration is shown in the bar graph, and error bars are provided.

extinction coefficient of GS-441524 in MilliQ water at 240 nm is 31,710 $M^{-1}cm^{-1}$ and at 245 nm is 25,336 $M^{-1}cm^{-1}$, within 5% of the published extinction coefficient of remdesivir triphosphate at 245 nm of 24,100 $M^{-1}cm^{-1}$ published in the SI of Dangerfield et al.[27] We used this extinction coefficient to calculate the concentration of each vial of GS-441524 seen in table 1. We did not receive duplicates of every brand, but we did receive multiple vials of Oscar, Panda, Shire, and Trusted, and we found that most of the concentrations did not vary dramatically between vials - the standard deviations were all less than 1 mg/mL. The highest difference was seen in Panda with a standard deviation of 0.88 mg/mL. Shire had a standard deviation of 0.71 mg/mL, and the variation was lowest with Oscar at 0.55 mg/mL.

For 14 out of 17 of the brands tested, the measured concentrations were within 10% of the concentration claimed by the company. For 3 of the brands, Oscar, Panda and Karma the concentrations were over 10% higher than advertised: Karma was 12% higher, Oscar was 18% higher, and Panda was 22%. Overconcentration has long been suspected of most brands and we found it only in three out of 17 brands. Comparisons

of the observed concentration compared to the manufacturer's claimed concentrations are shown in Figure 1B.

In contrast, one brand was dramatically under concentrated. The Shire brand was donated to our work with a note on the box from the donors that said, "this kills cats" however the donors did not know why or give us data to support this assertion. We received over 50 vials and chose 8 vials to be tested at random. We found the Shire vials to be consistently under concentrated, averaging nearly 50% below the marketed value. The fact that the Shire vials were ineffective was discovered quickly upon usage by the administrators of the crowdsource groups because the treated cats did not recover at a similar rate to the cats in the Pedersen efficacy studies.[26] We next made an effort to characterize several of the vials we had duplicates from Shire, Panda (Maxpaw), and Oscar to determine if the components of the vials were the same.

We first used high-performance liquid chromatography to purify the GS-441524 from the diluents and to determine purity. Each run of the crowdsource vials contained a major peak consisting of over 80% the area under the curve at 220 nm. The masses of the major peaks from Shire, Panda, Oscar, and the positive control had an m/z of 292.105 which matched the expected protonated mass of 292.10 seen in Figure S4. To validate the mass spec data, we also ran NMRs of the Shire, Panda and positive control samples. The samples were prepared in differently, so the proton peaks were shifted between samples seen in Figure S5. However, HSQC results seen in Figure S6 show similar patterns in the proton C13 correlations between samples indicating to us that these were likely all GS-441524 in different buffer conditions.

We also found a minor peak that consisted of less than 5% of the total area. The minor peak had a retention time of 7.62 minutes, compared to the average of 6.34 minutes for GS-441524 seen in table 2. This impurity absorbs at 240 nm like GS-441524, so the concentration measurements shown in table 1 were slightly inflated. Table 2 shows the concentrations corrected for percent purity at 240 nm. The fractions containing the impurity were found to have a larger m/z of 328 g/mol and can be seen for Shire, Panda, and Oscar in Figure S7. This impurity was contaminated with PEG even after HPLC purified. HPLC purified and lyophilized peak 2 was a transparent liquid as if polyethylene glycol was still present, and the results found in NMR analyses of the liquid were indicative of PEG. The percentage of the impurity measured at 220 nM ranged from 0.6% in Trusted to 4.2% in Panda with similar proportions when measured at 240 nm but were smaller ranging from 0.7% to 4.7% seen in table 2

Brand	Advertised concentration	240 nm Purity (%)	Calculated Concentration corrected for 240 nm purity	Percent major impurity (220 nm)	Percent major impurity (240 nm)	Impurity peak retention time
Shire	15 mg/mL	88.5	7.73 mg/mL	3.3	3.5	7.304
Seka	15 mg/mL	95.5	13.8 mg/mL	2.0	2.0	7.557
Trusted	15 mg/mL	91.5	13.5 mg/mL	4.3	4.2	7.613
Lucky 15	15 mg/mL	98.1	15.7 mg/mL	1.1	1.0	7.717
Oscar	15 mg/mL	95.3	16.9 mg/mL	0.9	1.0	7.740
Valor	17 mg/mL	97.3	17.3 mg/mL	1.2	1.2	7.631
Panda	15 mg/mL	94.0	17.2 mg/mL	4.2	4.1	7.648
Rainbow	20 mg/mL	97.4	19.4 mg/mL	0.6	0.7	7.700
Ohana	20 mg/mL	97.4	19.4 mg/mL	0.9	0.9	7.686
Karma	18 mg/mL	96.8	19.5 mg/mL	1.0	1.1	7.622
Ambeed	n/a	95.3	n/a	0.9	1.0	7.740

Table 2. HPLC purity analysis of 10 brands of unregulated GS-441524 and a positive control from Ambeed. The percent purity at 240 nm was used to calculate the corrected, and more accurate concentrations. Next is shown percent of the major impurity recorded at either 220 nm or 240 nm, and finally the impurity retention time..

Comparison of GC376 vials

We received 5 GC376 vials which we labeled JJ1 through JJ5. JJ1 and JJ2 were reportedly older while JJ3, JJ4 and JJ5 were all from the same newer batch. An inventory and photographs of these vials is shown in Figure S8. The absorbance spectra of all five vials are shown in Figure 2a. One spectrum, JJ1 looked more similar to GS than GC as shown in Figure 2a. To further characterize JJ1 we applied aliquots of JJ1 and JJ2 to Kim wipes and used a UV lamp to measure fluorescence and JJ1 fluoresced while JJ2 did not, as seen in Figure S10. The fluorescence spectrum of JJ1 is comparable to the fluorescence spectra of our GS-441524 positive control with a fluorescence maximum at 420 nm when excited at 250 nm. In addition, pH of JJ1 was

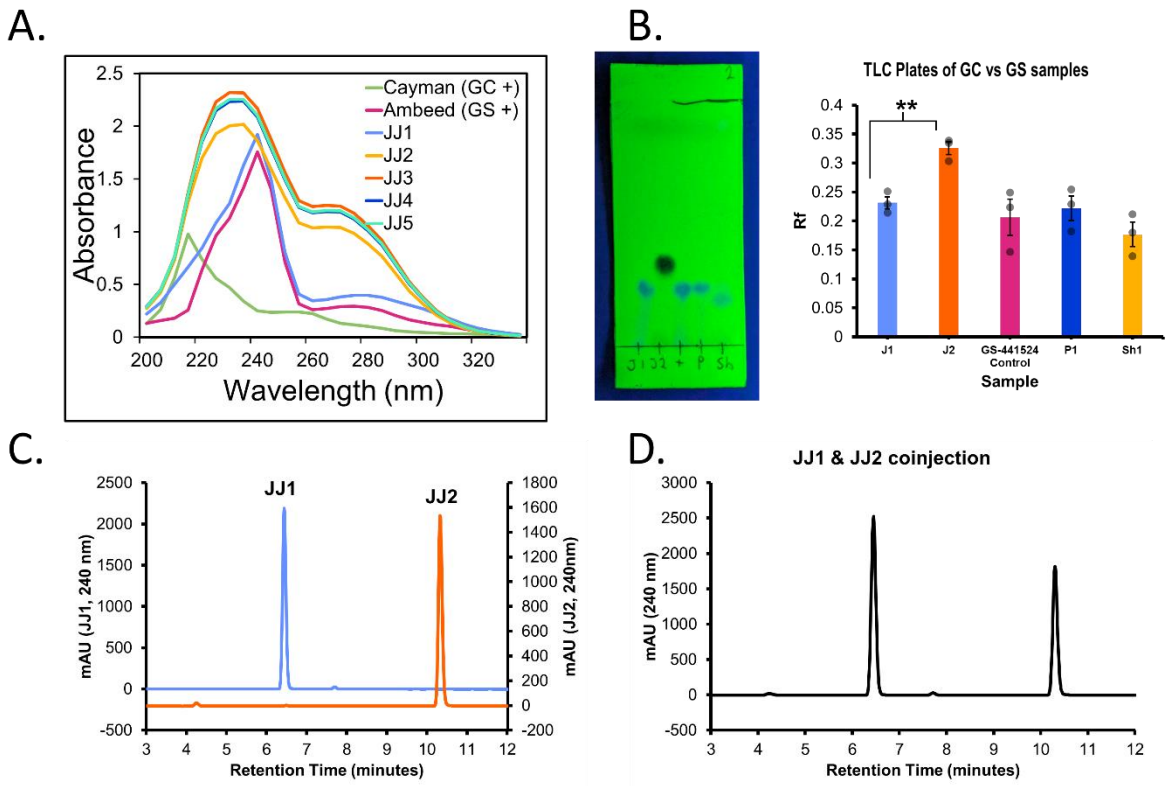


Figure 2. Comparison vials of GC-376. A. Line graph showing the absorbance spectra of all 5 vials of GC376, labelled JJ1-JJ5, and the positive control GS-441524. B. Sample TLC plate developed in ethy acetate comparing JJ1 and JJ2 to the positive control, Panda (P or P1) and Shire (Sh or SH1). C. HPLC trace measured at 240 nm showing JJ1 in blue and JJ2 in orange. D. An HPLC trace measured at 240 nm showing the coinjection of 2:1 JJ1 to JJ2.

1.87 is under 2 just like the GS-441524 samples. We ran TLC plates on JJ1 and JJ2 along with the GS-441524 positive control and samples of Panda and shire seen in Figure 2b and Figure S11.

Vial	pH	Retention time	Retention factor
JJ1	1.87	6.436	0.03
JJ2	5.97	10.323	0.2
JJ3	7.10	10.095	0.2
JJ4	7.17	10.105	0.2
JJ5	7.25	10.164	0.2

Table 3. Results of testing 5 vials of unregulated GC376. The retention factor was calculated using TLC plates developed in pure ethyl acetate.

JJ1 fluoresced like the GS-441524 and had a comparable retention factor of 0.025 whereas JJ2 had a larger retention factor of 0.186 and was not fluorescent. We ran HPLC and the traces of JJ1 alone, JJ2 alone, and JJ1 and JJ2 combined seen in Figure 2c and 2d. JJ1 has a retention time of 6.43 minutes comparable to that of GS average of 6.34 minutes and the major peak of JJ2 has a retention time of 10.3. The pH, retention times and retention factors are shown in Table 3. Finally, the M/Z of JJ1 was 291.104 which matched the positive control for GS-441524 seen in Figure S12. The concentration of GS-441524 in JJ1 was calculated to be 10.7 mg/mL.

The mischaracterization of JJ1 called into question the identity of JJ2-5. We obtained a standard for GC376 from Cayman Chemical to use as a standard. The HPLC traces of the GC376 standard compared to all the JJ vials were markedly different seen in Figure 3A. The GC376 standard displays 2 major peaks which is to be expected due to the spontaneous loss of the sulfate moiety as is expected in aqueous solutions and formation of the aldehyde which creates a mixture of stereoisomers due to epimerization.[28] This was confirmed by mass of the positive control with an expected m/z of 404 as shown in Figure S13. The published lambda max for GC376 is 206 nm in

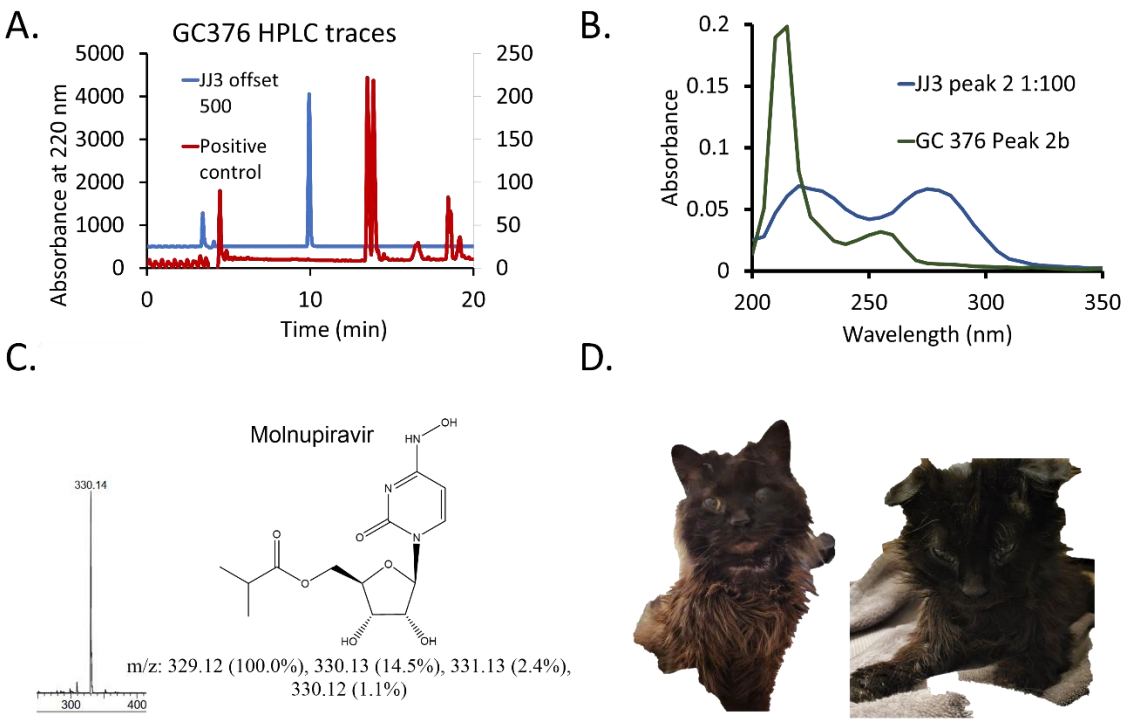


Figure 3. Characterization of vials of GC376. A. Line graph showing the absorbance spectra of JJ2 positive control GC376. the JJ3 axis is on the left and the positive control axis is on the right. B. HPLC traces measured at 220 nm comparing JJ3 and the GC376 positive control. JJ3 is offset by 500 absorbance units. C. Line graph showing the absorbance spectra of fractions from the HPLCE. In green is the positive control GC 376 peak 2b that eluted at 14 minutes and in blue is JJ peak 2 diluted 1:100 that eluted at 10 minutes. D. The mass spectrum peak of JJ2 peak 2 and the corresponding structure of molnupiravir. E. Photographs of a cat before and after treatment with these GC376 medications.

NaCl, which is similar to the λ_{max} of the major peak of our GC376 standard at 215 nm in MilliQ water [28]. However, the absorption spectra for the major JJ peak showed 2 peaks which do not align with any published GC376 spectra seen in Figure 3B. However, another antiviral drug, Molnupiravir (EIDD-2801), does have a similar bimodal absorption spectrum.[29] The m/z for JJ2 was 330.14 seen in Figure 3C. This corresponds to the mass of Molnupiravir+H^+ confirming that the JJ compounds were indeed Molnupiravir rather than GC376. Molnupiravir has shown efficacy against COVID-19 and has also been used in felines with FIP, but has demonstrated characteristic side effects including, “folded ears, losing whiskers, and severe leukopenia”.[20] The fact that the “GC376” vials were actually mislabeled samples of

Molnupiravir explains the observation of these side effects in cats treated with the vials, as seen in Figure 3D.

Discussion

FCoV is a cat coronavirus that affects the gastrointestinal tract of cats and is endemic in cat populations. In a subset of cats, especially cats that are young or with weakened immune systems, FECV can mutate into FIPV which is soon after fatal and can be devastating to pet owners. However, new antiviral drugs have been discovered that show efficacy against FIPV [13,26]. The most common of these is GS-441524, the active metabolite of remdesivir, but there are additional second tier drugs such as GC376 and Molnupiravir which have also shown efficacy against FIPV.

Despite the clearly demonstrated efficacy and a high demand from pet owners and feline conservation efforts, GS-441524 is not FDA approved and Gilead has reportedly refused to license GS-441524 for use in cats.[30] Anivive Lifesciences has a licensing agreement with Kansas State University and are actively making progress towards getting GC376 approved for use in cats.[31] However, the process can take years and in the meantime cats are diagnosed with FIP and the only path towards a cure is to acquire these medications without FDA approval through one of several crowdsource groups that help people access these drugs and mentor their clients through dosage, administration, and testing of these drugs. However, these drugs arrive with limited characterization and quality control, so we sought to determine the components and purity of these medications.

In this work we tested over 30 vials with 17 different labels of GS-441524 and 5 vials of GC376. For the GS-441524 vials tested, all of the pH values were under 2 which is required for stability and solubility of GS-441524. The absorbance peaks closely matched the positive control GS-441524. The concentrations measured in 13/17 vials were also comparable to the concentrations advertised by the manufacturer, in contrast with the widely held assumption that all unregulated GS-441524 vials were overconcentrated. 3/17 vials were between 10-25% overconcentrated. However, GS-441524 has a large safety profile called “impressive” by Dr. Pedersen who first tested these drugs[26]. Some toxicity was seen in one of the cats tested by Dr. Pedersen, and it is possible that a 25% overconcentration could cause unnecessary toxicity in treated cats.[26] 1/17 vials, from a manufacturer called Shire was known by the administrators of the crowdsource groups to be ineffective, and was found to be almost half the concentration claimed by the manufacturers. Cats given medication from these vials often relapsed and it was assumed an impurity in these vials was the cause but according to our data the components of the shire brand were not particularly different from the other brands tested. According to the administrators, cats treated with Shire, “reacted like taken off meds.” and we hypothesize that likely due to the lower concentration resulted in a dose that was below the therapeutic window and that was causing the cats to relapse.

The administrators of the crowdsource groups used grades of quality from “good” to “bad” for GS-441524 based on how effective each brand was in cats. We sought to find if there was a measurable characteristic that correlated with their perceptions of quality. According to the administrators I spoke with, “we don’t use Trusted, Panda,

Seka, [and] obviously Shire". For Trusted it was attributed to the exceptionally low pH that we also observed, making it difficult to use. In addition, they said, "Karma and Valor are good but considered "budget brands". We found the factor that correlated best with their field observations of quality was the percent purity of these brands. We observed a common impurity in all the unregulated GS-441524 vials tested that was not seen in our positive control GS-441524. This impurity proved difficult to characterize but closely corresponded with the field perceptions of quality. The order from most pure to least pure in our studies were Rainbow, Ohana, Lucky15, Oscar, Karma, Valor, Seka, Shire, Panda, Trusted. The top 6 all have less than 1.2% impurity and were all considered trustworthy, however the bottom 4 were advised against. It's possible that some cats react to this impurity or that the impurity means the overall concentration is low enough to dip below the therapeutic window. To confirm which is happening more research is needed to confirm the identity of the impurity.

In addition to the GS-441524 vials we analyzed 5 vials purported to contain 53 mg/mL of GC376. The first and oldest vial was found to contain GS-441524 as determined by HPLC retention time, fluorescence, absorbance, and mass spectrometry. The concentration was 10.7 mg/mL. GC376 is commonly used as a second-line, add-on therapy for cats unresponsive to GS-441524 alone. GS-441524 is most commonly used at a dosage of 10 mg/kg but doses in cats unresponsive to lower doses GS-441524 have been treated at as high as 50 mg/kg. The addition of 6-10 mg/kg GS-441524 from the mislabeled GC376 could result in unnecessary toxicity. GS0441524 has limited solubility in water and can precipitate causing stones.[32]

This result called into question the other JJ vials. We determined using a GC376 standard that these vials were also not what the manufacturer claimed they were. We determined through retention times, UV-vis absorbance, mass spectrometry, and reported effects on cats that GC vials JJ2-JJ5 instead contained a third antiviral, Molnupiravir. Molnupiravir has shown efficacy against coronaviruses, but it works on the same enzyme as GS-441524 [33], and so therefore it is unknown if FIPV that is resistant to GS-441524 will be susceptible to Molnupiravir. These substitutions can result in unnecessary risk to cats but are an intrinsic hazard of purchasing unregulated drugs.

We sought to find ways to help administrators and pet owners to characterize these antivirals at home. GS-441524 was easy to distinguish from the others. GS-441524 is fluorescent and can be visualized under a black light – we used tissue paper seen in supplemental figure 9. In addition, pH measurements can be helpful - GS-441524 requires a much lower pH than the other drugs for solubility and stability. While those techniques are helpful, none are conclusive, and we found the best characterization technique sans expensive equipment was done with thin layer chromatography (TLC).

We found that TLC was the most reliable, versatile, and facile method for qualitatively assessing the components of the vials. This technique can be done with no special equipment but is enhanced by access to a short-wave UV lamp. Our TLC technique is described in depth in the supplemental appendix A. The TLC plate doesn't even have to be developed to give informative results. GS-441524 is fluorescent and its

emission can be seen under low energy UV light, ours was 365 nm. To distinguish GC376 from Molnupiravir, a TLC plate with fluorescent backing is required as well as a high-energy UV light, ours was 254 nm, and Molnupiravir absorbs the light well resulting in a dark spot while GC376 does not absorb well at those wavelengths and is nearly invisible seen in Appendix A. These differences are even more apparent when the TLC plates are developed in ethanol. We used 100% ethanol to develop the TLC plates and dilute drug to prevent smearing, though the 95% ethanol that can be purchased at the store will also work. While the best practices require LC or HPLC with standards, or better yet import with FDA approval and regulations, this technique can be used to roughly characterize and distinguish these 3 antivirals.

Conclusions

In conclusion, people who want to save the life of their pets sometimes turn to experimental drugs that are not FDA approved or regulated and with that comes risks. We found that most, 13/17, of the GS-441524 brands tested had concentrations like the manufacturer's claims. Three of the 17 tested were slightly overconcentrated but given the large safety profile for GS-441524 that isn't the most concerning. 1/17 was nearly 50% under concentrated and that is concerning and resulted in cats treated with this brand relapsing and showing symptoms again probably due to the lower dose they were receiving (personal communication). However, the crowdsource group administrators knew this brand was ineffective. The administrators of the crowdsource group also organized the brand by quality that was closely correlated to the concentration of an impurity in the vials. Our conclusion is that while the testing methods may be

unconventional the administrators have a good approximation of the quality and components of the vials of GS-441524 that we tested.

This was not the case, however, with the GC376 vials we tested. GC376 is used as an additive drug when GS-441524 treatment is ineffective, and while GC376 has been shown to be active against coronaviruses, none of the vials tested contained GC376. One vial contained GS-441524 and 4 contained Molnupiravir.

Based on the data presented here, it is clear that the quality of the unlicensed FIP treatments available through crowdsourcing varies substantially. While many of the samples of GS-441524 contained the correct compound at very near the concentration advertised, some samples were more dilute or concentrated than expected. On the other hand, none of the samples of GC376 contained the reported drug. Here, we offer a few straightforward approaches to assessing the quality of the samples available, which can be used prior to using these medications in cats.

References

[1] Stout, A.E. et al. (2020). Coronaviruses in cats and other companion animals: Where does SARS-CoV-2/COVID-19 fit? *Veterinary Microbiology*. DOI: 10.1016/j.vetmic.2020.108777.

[2] Mesel-Lemoine, M. et al. (2012). A Human Coronavirus Responsible for the Common Cold Massively Kills Dendritic Cells but Not Monocytes. *Journal of Virology*. DOI: 10.1128/JVI.00269-12.

[3] Esper, F.P. et al. (2023). Alpha to Omicron: Disease Severity and Clinical Outcomes of Major SARS-CoV-2 Variants. *The Journal of Infectious Diseases*. DOI: 10.1093/infdis/jiac411.

[4] Guo, R. et al. (2023). When COVID-19 sits on people's laps: A systematic review of SARS-CoV-2 infection prevalence in household dogs and cats. *One Health*. DOI: 10.1016/j.onehlt.2023.100497.
[5] Duijvestijn, M.B.H.M. et al. (2023). Serological Survey of Retrovirus and Coronavirus Infections, including SARS-CoV-2, in Rural Stray Cats in The Netherlands, 2020–2022. *Viruses*. DOI: 10.3390/v15071531.
[6] Ratti, G. et al. (2022). Molecular Detection of Feline Coronavirus in Captive Non-Domestic Felids from Zoological Facilities. *Animals : an Open Access Journal from MDPI*. DOI: 10.3390/ani12141864.
[7] Stephenson, N. et al. (2013). Feline infectious peritonitis in a mountain lion (Puma concolor), California, USA. *Journal of Wildlife Diseases*. DOI: 10.7589/2012-08-210.
[8] Poland, A.M. et al. (1996). Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. *Journal of Clinical Microbiology*. DOI: 10.1128/jcm.34.12.3180-3184.1996.
[9] Vennema, H. et al. (1998). Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology*. DOI: 10.1006/viro.1998.9045.
[10] Pedersen, N.C. et al. (2008). Pathogenesis of feline enteric coronavirus infection. *Journal of Feline Medicine and Surgery*. DOI: 10.1016/j.jfms.2008.02.006.
[11] Pedersen, N.C. (2009). A review of feline infectious peritonitis virus infection: 1963–2008. *Journal of Feline Medicine and Surgery*. DOI: 10.1016/j.jfms.2008.09.008.
[12] Addie, D.D. (2019). Feline infectious peritonitis: answers to frequently asked questions concerning FIP and coronavirus. *Veterinary Nursing Journal*. DOI: 10.1080/17415349.2019.1629366.
[13] Murphy, B.G. et al. (2018). The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. *Veterinary Microbiology*. DOI: 10.1016/j.vetmic.2018.04.026.
[14] Ritz, S. et al. (2007). Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. *Journal of Veterinary Internal Medicine*. DOI: 10.1892/06-302.1.
[15] Cihlar, T. and Mackman, R.L. (2022). Journey of remdesivir from the inhibition of hepatitis C virus to the treatment of COVID-19. *Antiviral Therapy*. DOI: 10.1177/13596535221082773.
[16] Warren, R. (2022). https://www.vetmed.ucdavis.edu/news/clinical-trial-places-cats-fip-remission.
[17] Krentz, D. et al. (2021). Curing Cats with Feline Infectious Peritonitis with an Oral Multi-Component Drug Containing GS-441524. *Viruses*. DOI: 10.3390/v13112228.
[18] Jones, S. et al. (2021). Unlicensed GS-441524-Like Antiviral Therapy Can Be Effective for at-Home Treatment of Feline Infectious Peritonitis. *Animals*. DOI: 10.3390/ani11082257.
[19] Sharun, K. et al. (2020). Protease inhibitor GC376 for COVID-19: Lessons learned from feline infectious peritonitis. *Annals of Medicine and Surgery*. DOI: 10.1016/j.amsu.2020.12.030.

[20] Roy, M. et al. (2022). Unlicensed Molnupiravir is an Effective Rescue Treatment Following Failure of Unlicensed GS-441524-like Therapy for Cats with Suspected Feline Infectious Peritonitis. *Pathogens*. DOI: 10.3390/pathogens11101209.
[21] Lv, J. et al. (2022). Effect of GS-441524 in combination with the 3C-like protease inhibitor GC376 on the treatment of naturally transmitted feline infectious peritonitis. *Frontiers in Veterinary Science*.
[22] Lu, J. et al. (2022). Crystallization of Feline Coronavirus Mpro With GC376 Reveals Mechanism of Inhibition. *Frontiers in Chemistry*.
[23] Dampalla, C.S. et al. (2021). Postinfection treatment with a protease inhibitor increases survival of mice with a fatal SARS-CoV-2 infection. *Proceedings of the National Academy of Sciences of the United States of America*. DOI: 10.1073/pnas.2101555118.
[24] Luan, X. et al. (2023). Structure basis for inhibition of SARS-CoV-2 by the feline drug GC376. *Acta Pharmacologica Sinica*. DOI: 10.1038/s41401-022-00929-z.
[25] Zhang, S. (2020). https://www.theatlantic.com/science/archive/2020/05/remdesivir-cats/611341/.
[26] Pedersen, N.C. et al. (2019). Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. *Journal of Feline Medicine and Surgery*. DOI: 10.1177/1098612X19825701.
[27] Dangerfield, T.L. et al. (2020). Remdesivir Is Effective in Combating COVID-19 because It Is a Better Substrate than ATP for the Viral RNA-Dependent RNA Polymerase. *iScience*. DOI: 10.1016/j.isci.2020.101849.
[28] Vuong, W. et al. (2021). Improved SARS-CoV-2 Mpro inhibitors based on feline antiviral drug GC376: Structural enhancements, increased solubility, and micellar studies. *European Journal of Medicinal Chemistry*. DOI: 10.1016/j.ejmech.2021.113584.
[29] Dömötör, O. and Enyedy, É.A. (2023). Evaluation of In Vitro Distribution and Plasma Protein Binding of Selected Antiviral Drugs (Favipiravir, Molnupiravir and Imatinib) against SARS-CoV-2. *International Journal of Molecular Sciences*. DOI: 10.3390/ijms24032849.
[30] https://www.aaha.org/publications/newstat/articles/2022-11/fip-research-new-hope-for-cats-and-maybe-humans/.
[31] King, I. (2018). https://consciouscat.net/fip-treatment-waiting-for-fda-approval/.
[32] (2023). https://vetmed.umn.edu/urolith-center/image-of-month/mysterious-new-stone-type-cats.
[33] Hashemian, S.M.R. et al. (2022). RdRp inhibitors and COVID-19: Is molnupiravir a good option? *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*. DOI: 10.1016/j.biopha.2021.112517.