The long history of Beta-d-N4-hydroxycytidine and its modern application to treatment of Covid-19 in people and FIP in cats.

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Beta-d-N4-hydroxycytidine is a small molecule (nucleoside) that was studied in the late 1970s in the old Soviet Union as part of biological weapons research [2]. Weaponization of diseases like smallpox was a worldwide fear, but the dangers of using smallpox virus for this purpose was just too great. Smallpox had been eradicated from the world, virtually all stocks destroyed, and further research forbidden. This led to research by both the US and the Soviet Union into other RNA viruses as biological weapons and antivirals to defend against them. Venezuelan equine encephalomyelitis virus (VEEV) was one of the first viruses seriously considered as a biological weapon [3]. VEEV is transmitted to humans through mosquito bites, and causes high fever, headaches, and encephalitis, and swelling of the that can be deadly. Beta-d-N4-hydroxycytidine was found not only to inhibit VEEV replication, but a broad range of alphaviruses, including Ebola, chikungunya, influenza virus, norovirus, bovine diarrhea virus, hepatitis C virus and respiratory syncytial virus. [3-8]. The earliest report of the inhibitory effect of beta-d-N4-hydroxycytidine on a human coronavirus NL63 was in 2006 [9]. Recent studies have confirmed its inhibitory effect on a broad range of human and animal coronaviruses [8].

An important part of the more recent history of beta-d-N4-hydroxycytidine comes from the Emory Institute for Drug Development (EIDD) [1], and its experimental designation was EIDD-1931. Significant financial support for studies of antivirals against alphaviruses at institutions like Emory was provided by the US government as far back as 2004, with considerable financial support to present [10]. The Defense Threat Reduction Agency provided institutional support in 2014 with the goal to find an antiviral compound against VEEV and other alphacoronaviruses. “N4-Hydroxycytidine and derivatives and anti-viral Uses related thereto” was covered by US Patent Application 2016/106050 A1 in 2016 [11]. Additional funding came in 2019 from the National Institute of Allergy and Infectious to partner research into an esterified prodrug of beta-d-N4-hydroxycytidine (EIDD-2801) for treatment of influenza [10]. The stated intent of the chemical alterations to EIDD-2801 was to enhance its oral bioavailability, which would ultimately allow beta-d-N4-hydroxycytidine to be administered as pills rather than injections. A switch of research emphasis from influenza to SARS-II CoV came in 2019/2020 [2]. Commercialization of EIDD-2801 was given to an Emory Affiliate called Ridgeway Biotherapeutics, which then partnered with Merck for the lengthy and expensive FDA approval process. The current field-testing version of EIDD-2081 has been named Molnupiravir.

Beta-d-N4-hydroxycytidine, the active ingredient of Molnupiravir, exists in two forms as tautomers. In one form, it mimics cytidine, with a single bond between the carbon and N-OH group. In its other form, which mimics uridine, it has an oxime with a double bond between the carbon and N-OH group. In the presence of beta-d-N4-hydroxycytidine, the viral RNA-dependent RNA polymerase reads it as uridine instead of cytidine and inserts an adenosine instead of guanosine. Switching between forms causes mismatching during transcription, resulting in numerous mutations in the viral genome, and cessation of viral replication [8].

Advancement of Molnupiravir by Merck to conditional and full approval by the FDA is on an accelerated course. In a corporate statement, Merck has stated the following [12] - *“In anticipation of the results* 2

from MOVe-OUT, Merck has been producing Molnupiravir at risk. Merck expects to produce 10 million courses of treatment by the end of 2021, with more doses expected to be produced in 2022. ……. . Merck is committed to providing timely access to Molnupiravir globally, if it is authorized or approved, and plans to implement a tiered pricing approach based on World Bank country income criteria to reflect countries' relative ability to finance their health response to the pandemic. …… . As part of its commitment to widespread global access, Merck previously announced that the company has entered into non-exclusive voluntary licensing agreements for Molnupiravir with established generic manufacturers to accelerate availability of Molnupiravir in more than 100 low- and middle-income countries (LMICs) following approvals or emergency authorization by local regulatory agencies." It is unlikely that this "generosity" will also apply to animal use.

Drugs to inhibit the causative agent of the current Covid-19 pandemic, have been rapidly field tested over the last two years and one of them, Remdesivir, was approved in record time for hospitalized patients. Molnupiravir has been moved toward conditional approval within the last year as an oral drug for home treatment of early-stage infection [12]. However, effective anti-coronavirus compounds were developed earlier for another common and highly disease of cats, feline infectious peritonitis (FIP). These drugs include a protease inhibitor (GC376) [13] and an RNA dependent RNA polymerase inhibitor (GS-441524) that is the active moiety of Remdesivir [14]. The success in treating FIP with antiviral drugs has prompted a recent study of both EIDD-1931 and EIDD-2801 for their ability to inhibit FIPV in tissue culture [15]. The effective concentration-50% (EC50) for EIDD-1931 against FIPV is 0.09 uM, EIDD-2801 0.4 uM and GS-441524 0.66 Um [15]. The percent cytotoxicity at 100 uM is 2.8, 3.8 and 0 for these compounds, respectively. Therefore, EIDD-1931 and -2801 are slightly more virus inhibitory but also more cytotoxic than GS-441524. These laboratory studies indicate that both EIDD-1931 and EIDD-2801 are also excellent candidates for FIP treatment.

Although EIDD-1931 and EIDD-2801 hold great promise for the treatment of FIP, there are several obstacles that make legal use of these compounds unlikely any time soon. GS-441524, the active form of Remdesivir, and patented by Gilead Sciences, was researched for use in cats with FIP shortly before the Covid-19 pandemic occurred. Therefore, it was the potential use of Remdesivir against Ebola virus and not SARS-like coronaviruses that prompted research on FIP [14]. Even though these studies were done in collaboration with scientists from Gilead Science, the company refused animal rights for GS-441524 once it became obvious that there was a much larger market for Covid-19 in humans [16]. Similarly, my attempts over the last 2-3 years to Emory, Ridgeback Biotherapeutics, and the Veterinary Division of Merck to research EIDD-1931 and EIDD-2801 for FIP in cats have either gone unanswered or rebuffed, undoubtedly for similar reasons to why Gilead refused to grant animal rights for GS-441524. However, the great worldwide need for an FIP treatment rapidly fueled an unapproved market for GS-441524 out of China. This same need to treat FIP has recently fueled interest on Molnupiravir as a treatment for FIP, also out of China.

The situation with EIDD-1931 and EIDD-2801/Molnupiravir and GS-441524 and Remdesivir brings to question why some drugs are converted to prodrugs for marketing [17]. Remdesivir was reportedly esterified to increase antiviral activity, although studies in cats showed that GS-441524 and Remdesivir had similar virus inhibitory activity in tissue culture [18]. However, Remdesivir was found to be poorly absorbed by the oral route and was therefore conditionally approved only for injection. EIDD-2801 was created to enhance oral absorption of EIDD-1931, even though earlier research indicated that EIDD-1931 is well absorbed orally without esterification [6]. The motives behind the commercialization of Remdesivir instead of GS-441524 for use in humans has been scientifically questioned, as the latter appears to be superior in several ways without further modifications [17]. Why was EIDD-2801 was put

forward for commercialization when EIDD-1931 would be cheaper, 4 times more virus inhibitory and one third less toxic than EIDD-2801 [15]? Strength of patent rights and patent longevity may be more compelling factors in these decision [16, 17, 19].

One of the problems in the treatment of FIP in cats is the blood-to-eye and blood-to brain barriers, which become of great importance when the disease affects the eyes and/or brain [13, 14, 20]. This problem has been overcome in large part in the treatment of ocular and neurological forms of FIP with GS-441524 by progressively increasing the dosage to raise blood levels and therefore the concentration of drug in the aqueous humor and/or brain [20]. GC376, one of the most potent antivirals against FIP virus in culture [17], is not effective against ocular and neurological FIP because of the inability to get enough drug into these sites even when increasing the dosage several times [14]. Fortunately, it appears that EIDD-1931 can reach effective levels in the brain as indicated by studies in horses with VEEV infection [3]. Drug resistance is another problem that is now being seen in some cats being treated with GS-441524, especially individuals with the neurological form of FIP. The long treatment courses and difficulty in getting sufficient drug into the brain favors the development of drug resistance.
The short- and long-term toxic effect of a candidate drug on the test person or animal is of prime importance. GS-441524 has a lower toxicity than GC376, EIDD-1931 and EIDD-2801 in cell cultures [15]. However, it is the toxicity that occurs *in vivo* that is most important. GC376 is among the most coronavirus inhibitory drug known [15], but it will retard adult dentition when given to young kittens [13]. No serious toxicity has been observed over almost three years of field use of GS-441524, mirroring the complete lack of cytotoxic effects in vitro at concentrations as high as 400 uM [18]. However, EIDD-1931 and EIDd-2801 demonstrate significant cytoxicity at 100 uM [15]. Therefore, it is the ability of EIDD-1931 to create fatal mutations in RNA has been of greatest concern for some time [8, 21, 22]. This has been a big reason why it has been slow to be applied to disease. However, the current recommended treatment of Covid-19 with Molnupiravir is for only 5 days at the early stage of treatment [10]. However, the recommended treatment with GS-441524 for FIP is 12 weeks [14], allowing much greater time for toxicity to manifest itself. Therefore, it will be important to carefully observe cats on EIDD-1931 or EIDD-2801 treatment for both short- and long-term effects.

All antiviral drugs to date have yielded to the development of drug resistance through mutations in the viral genome. Although Remdesivir has appeared less susceptible to such mutations than other drugs used in viral diseases like HIV/AIDS, drug resistance has been well documented [23-26]. Resistance to GS-441524 in cats being treated for FIP has been seen with greater frequency, especially in cats with neurological FIP, where it is more difficult to get sufficient drug into the brain [13, 14, 20]. Resistance to GS-441524 in cats is also likely to be more of a problem because cats with FIP are often treated for 12 weeks or more, while Remdesivir (and Molnupiravir) are recommended for only five days during the initial viremic stage of Covid-19 [16]. The problem of drug resistance has been effectively managed in HIV/AIDS treatment by using a cocktail of different drugs at the same time with different resistance profiles. Drug resistant mutants to one drug will be immediately inhibited by the other drugs, preventing their positive selection in the face of treatment. Inhibition of resistance is particularly strong when the two drugs attack different proteins involved in virus replication. For instance, GC376 is a protease inhibitor [13], while GS-441524 acts on the RNA dependent RNA replicase [18]. However, GC376 is not as well absorbed across the blood-to-brain barrier. Although sufficient work has not yet been done, it appears that there will be no cross-resistance between GS-441524 and Molnupiravir but is as effective as GS-441524 in crossing the blood-to-brain barrier [3]. These things would make Molnupiravir (or 5-hyroxycytidine) and important addition to future FIP treatment.

As anticipated, Molnupiravir has recently been tested in cats with FIP by at least one Chinese sellers of GS-441524, and preliminary results reported on the FIP Warriors CZ/SK website [27]. The field trial consisted of 286 cats with various forms of naturally occurring FIP seen in pet clinics in US, UK, Italy, Germany, France, Japan, Romania, Turkey, and China. No deaths occurred among 286 cats that participated in the trial, including seven cats with ocular (n=2) and neurological (n=5) FIP. Twenty-eight of these cats were cured after 4-6 weeks of treatment and 258 after 8 weeks. All treated cats remained healthy 3-5 months later, a period during which relapses would be expected in cats not successfully cured. This data provides compelling evidence for the safety and efficacy of Molnupiravir for cats with various forms of FIP. However, it is hoped that this field trial will be written in manuscript form, submitted for peer review, and published. Nevertheless, it is now being sold to owners of cats with FIP. At least one other major seller of GS-441524 is also interested in using Molnupiravir for FIP, indicating a demand for additional antiviral drug treatments to cats with FIP.

A safe and effective dosage for Molnupiravir in cats with FIP has not been published. However, at least one seller out of China has provided some pharmacokinetic and field-testing data on Molnuparivir in cats with naturally occurring FIP in their advertising flier for a product called Hero-2081 [27]. However, this information does not clearly state the amount of Molnupiravir in one of their “50 mg tablets” and the actual dosing interval (q12h or q24h?). Fortunately, an estimated starting dosage for Molnupiravir in cats with FIP can be obtained from published in-vitro cell culture studies of EIDD-1931 and EIDD-2801 [15] and laboratory and field studies of GS-441524 [14,18]. Molnupiravir (EIDD-2801) has an EC50 of 0.4 uM/ul against FIPV in cell culture, while the EC50 of GS-441524 is around 1.0 uM/ul [18]. They both have similar oral absorptions of around 40-50%, so an effective subcutaneous (SC) dosage for Molnupiravir would be approximately one-half the recommended 4 mg/kg SC q24h starting dosage for GS-441524 [14], or 2 mg/kg SC q24h. The *per-os* (PO) dosage would be doubled to account for less efficient oral absorption to a dosage of 4 mg/kg PO q24h. An estimated starting oral dosage for Molnupiravir in cats with FIP can also be calculated from available data on Covid-19 treatment. Patients being treated for Covid-19 are given 200 mg of Molnupiravir PO q12h for 5 days. This dosage was obviously calculated from a pharmacokinetic study done on people, and if an average person weighs 60-80 kg (70 kg), the effective inhibitory dosage is ~3.0 mg/kg PO q12h. A cat has a basal metabolic rate 1.5 times a human, and assuming equal oral absorption for both people and cats, the minimum cat dosage by this calculation would be 4.5 mg/kg PO q12 hr for cats with FIP and no eye or brain involvement. If Molnupiravir crosses the blood-to-eye and blood-to-brain barrier at equal efficiency to GS-441524, e.g., ~40% [3,18], the starting dosage would be increased ~2.5 times to ~12 mg/kg PO, q12 h allow for adequate penetration into aqueous humor and cerebrospinal fluid for cats with ocular or neurological FIP. The duration of treatment would be 10-12 weeks and monitoring of treatment response identical to GS-441524 [14, 20]. These recommendations are based on presumptions from published information and more experience with Molnupiravir in the field will be needed.

It is doubtful that Molnupiravir will prove safer and any more effective than GS-441524 for the treatment of FIP, but a third antiviral drug may could prove extremely helpful in preventing GS-441524 resistance (as a cocktail of antivirals with different resistance profiles) or in treating cats that no longer respond well to GS-441524. The big unknown is whether Molnupiravir will be free from long term toxicities, as the active ingredient, N4-hydroxycytidine, is an extremely potent mutagen [21] and the treatment time for FIP is much longer than for Covid-19 and chances of side-effects greater.

It is unfortunate that EIDD-1931 (N4-hydroxycytidine), the active ingredient of Molnupiravir, was not given greater consideration for treating COVID-19 than Molnupiravir. EIDD-1931 is 4 times more virus inhibitory than Molnupiravir (EC50 0.09 vs 0.4 uM) and the percent cytotoxicity is not significantly different between the two drugs, ranging from 0-2.8% across the range of 10 to 100 uM [15]. Toxicity appears to rise for both drugs only at 100 uM. GS-441524, by comparison has no toxicity even at 400 uM [18]. N4-hydroxycytidine is also efficiently absorbed by the oral route [3], something downplayed in the development of EIDD-2801 (Molnupiravir). This scenario is identical to that of GS-441524 and Remdesivir, with the latter being chosen for commercialization even though current research indicates the former would have been the best candidate [17].

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