Advantages of the parent nucleoside GS-441524 over remdesivir for Covid-19 treatment

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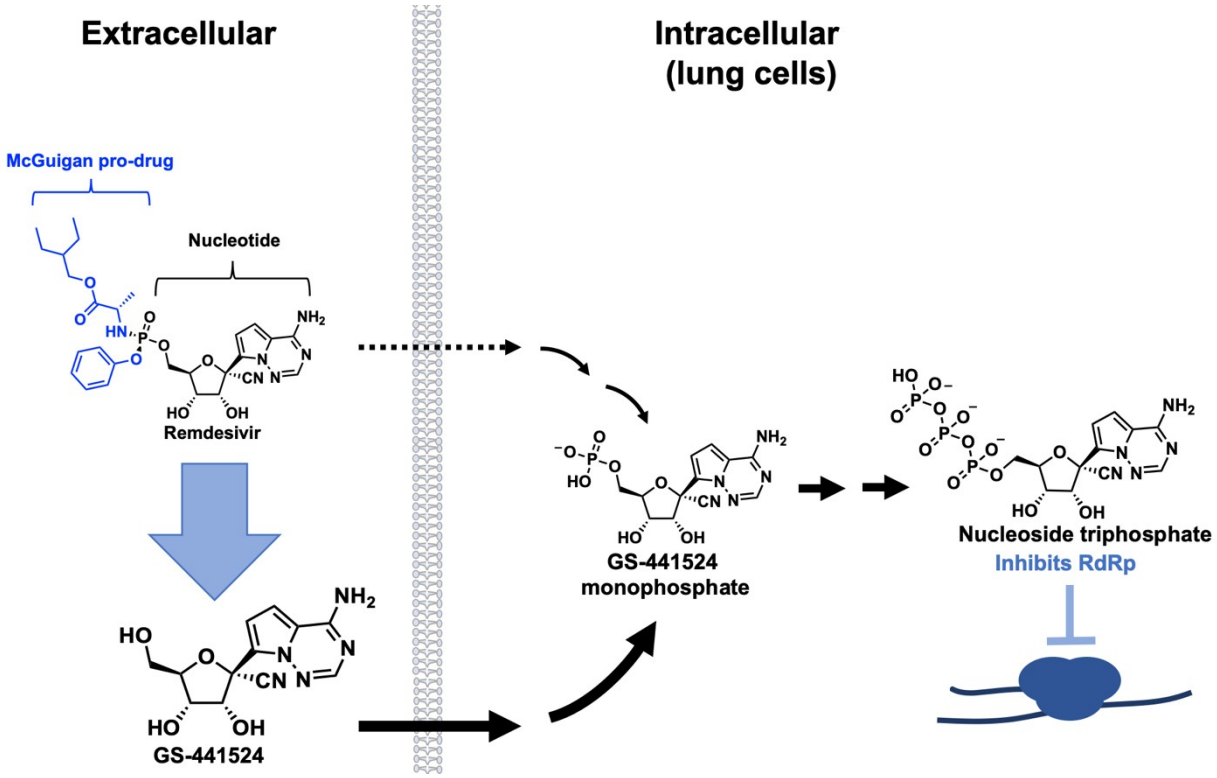

Abstract.

While remdesivir has garnered much hope for its moderate anti-Covid-19 effects in recent clinical trials, its parent nucleoside, GS-441524 has remained out of the spotlight despite exhibiting comparable potency in clinically relevant models of the lung. Our analysis of the in vivo pharmacokinetics of remdesivir evidences premature of hydrolysis of its phosphate pro-drug in serum such that GS-441524 is the predominant circulating metabolite that reaches the lungs. Under this broader pharmacokinetic rationale, we contend that GS-441524 is superior to remdesivir for Covid-19 treatment due to its synthetic simplicity, demonstrated in vivo potency against coronavirus models, and comparative ease of formulation into an inhalable prophylactic. Collectively, these advantages would simplify mass production and distribution and enable higher dosing of GS-441524 both therapeutically and prophylactically.

Introduction.

In December 2019, a series of respiratory outbreaks in Wuhan, China caused by severe acute syndrome respiratory coronavirus 2 (SARS-CoV-2) spurred the beginnings of an unprecedented global effort to counter the disease known as Covid-19[1,2]. With a plunge to the economic arms of countries worldwide also came a surge of genomic[1] and structural biology[3] efforts to understand the disease and enable therapeutic intervention. Several drugs used in other pathological contexts are currently being evaluated for the treatment of Covid-19 (NCT04323527, NCT04315298, NCT04252664). Promising amongst this growing list of re-purposed drugs is remdesivir. Originally developed by Gilead for the treatment of Ebola virus (EBOV)[4,5], remdesivir has shown broad spectrum activity against a variety RNA viruses, including SARS-CoV[6–11]. Recently, remdesivir has attracted much attention for encouraging data showing its ability to accelerate the time to recovery in patients with advanced Covid-19 in a controlled, NIH-sponsored clinical trial[12]. These encouraging data have prompted the US FDA to grant remdesivir emergency use authorization (EUA)[13] and for the Japanese Ministry of Health, Labour, and Welfare to approve it for the treatment of Covid-19[14]. Even in spite of these data, remdesivir has fallen short of its preclinical promise, which had suggested strong prophylactic and therapeutic efficacy in multiple *in vivo* models of coronavirus (MERS-CoV, SARS-CoV, SARS-CoV-2) such as mice[15] and primates[16,17]. Further undercutting the promising data with remdesivir in the NIH-sponsored clinical trial was the finding that it provided no statistically significant benefit to patients with severe Covid-19 in a similarly controlled clinical trial conducted in China[18]. Some may note that Chinese

clinical trial was not as well-powered as the one sponsored by the NIH, thus giving the results from the latter greater consideration. Still, the translation of the moderate efficacy observed in the NIH-sponsored trial is nevertheless impeded by the limited supply of remdesivir and its subsequently patchy distribution[19].

We recently described in a general audience publication[20] the advantages that the parent nucleoside of remdesivir, GS-441524, has over remdesivir itself for the treatment of Covid-19. Fundamentally, our investigation into the metabolism of remdesivir evidences premature hydrolysis of the phosphate pro-drug on remdesivir in serum, followed by dephosphorylation[15,16,21]. As a result, the major metabolite circulating in the bloodstream is the parent nucleoside, GS-441524, even though remdesivir (monophosphate nucleotide pro-drug) was the species initially administered. Accounting for this broader pharmacokinetic (PK) rationale, we herein provide a detailed analysis of the literature that supports the use of GS-441524 over remdesivir for the treatment of Covid-19.

The phosphate pro-drug on remdesivir is not intended for lung-specific delivery.

Remdesivir is a nucleotide monophosphate analogue of adenosine monophosphate (AMP) that interferes with RNA-dependent RNA polymerases[22]. As an AMP analogue, the anionic phosphate moiety on remdesivir is protected by McGuigan pro-drug moieties[23], (phenol and L-alaninate ethylbutyl ester) to increase lipophilicity and enhance cell permeability. In principle, these pro-drug moieties would be removed intracellularly—first by esterases (cathepsin A/carboxylesterase 1) and then by

phosphoramidases (HINT1)[9] to release the monophosphorylated nucleotide. This would then be phosphorylated twice to give the active NTP [9,22] (**Figure 1a**), which is substrate-competitive with ATP for incorporation by RNA polymerase—resulting in inhibition of viral RNA synthesis[22].

The combination of phenol and an amino acid ester as a phosphate (or phosphonate) pro-drug was first developed in 1992 by Christopher McGuigan[24] to enable delivery of a mono-phosphate nucleotide antiviral—overcoming the rate-limiting first phosphorylation step by thymidine kinase towards the active tri-phosphorylated species. Bioactivation of the pro-drug first involves carboxylesterases (CES1) and cathepsin A (CTSA), followed by phosphoramidases (histidine triad nucleotide binding proteins; HINTs; **Figure 1a**)[9,25,26]. Protein expression data from the Human Protein Atlas show that these enzymes (CES1, CTSA, HINT1, 2, 3) all have high expression in the liver, with minimal expression in type II pneumocytes in the lung[27] (**Figure 2**). For the HINT family of phosphoramidases, there is some slight variation in each isoform's tissue-specific expression (**Figure 2b, c**); however, all 3 isoforms show high expression in the GI tract, liver, and kidneys. From the pattern of bioactivation for McGuigan pro-drugs, it follows that the most significant accumulation active NTP will be in cell types with high expression of CES1/CTSA/HINT1-3, such as the liver. Preferential bioactivation of McGuigan pro-drugs such as remdesivir could explain the Grade 3/4 adverse events related to liver and kidney damage in Covid-19 patients treated with remdesivir[12]. Seeing that the enzymes involved in McGuigan pro-drug hydrolysis are hardly

expressed in the lungs undermines its utility in the context of a primarily respiratory disease such as SARS-CoV-2.

GS-441524 is the predominant metabolite in the bloodstream when remdesivir is administered intravenously.

Hydrolytic enzymes are ubiquitous in serum[28]. This is one physiological factor that prevents direct extrapolation of bioactivation mechanisms observed in cell-based systems to the *in vivo* setting—especially for pro-drugs[29]. For example, esterases and phosphatases are abundantly present in serum across species[30,31]. Premature hydrolysis of the McGuigan pro-drug on remdesivir in serum is thus unsurprising (**Figure 1b**). Multiple studies dating back to when remdesivir was being investigated for EBOV have demonstrated that the nucleoside, GS-441524, is the predominant species in serum after remdesivir is administered (**Figure 3b, c**)[15,16,21]. All studies that have investigated the PK of remdesivir in non-human primates (NHP) have concluded that intact remdesivir exhibits a short plasma half-life of about 0.4 hours in serum, with "persistence" of the downstream nucleoside, GS-441524 (**Figure 3c**)[16,21]. A recent study by Williamson and colleagues shows that, 24 hours after intravenous injection of remdesivir, GS-441524 is present in serum at concentrations 1000-fold higher than remdesivir throughout the 7-day treatment course[16] (**Figure 3b**). This recurring phenomenon can first be explained by the abundance of plasma esterases, as the phosphoramidases (HINT1) involved in removal of the L-alanine have a strictly intracellular presence (see Human Protein Atlas HINT 1-3). With this spatial considerations, inadvertent biotransformation of remdesivir to GS-441524 can be

explained by the following sequence of enzymatic steps: 1.) esterase removal of the L-alaninate ester, 2.) intramolecular cyclization, displacement of the phenolate, followed by re-opening of the ring, 3.) cleavage of the phosphate ester by serum phosphatases (**Figure 1b**). The proposed serum bioactivation mechanism accounts for the general substrate constraints for each class of enzyme. For instance, CES1 is named as one of the enzymes involved in McGuigan pro-drug hydrolysis—perhaps because the McGuigan pro-drug on remdesivir is the most optimal substrate for CES1. However, this does not preclude other esterases from acting on its L-alaninate ester. A study conducted by Sheahan and colleagues specifically investigated the PK of remdesivir in carboxylesterase 1c deficient mice (*Ces1c*$^{-/-}$)[15]. Even in this *Ces1c*$^{-/-}$ model, the half-life of remdesivir was still short ($t_{1/2}$ ~25 minutes;), supporting the notion that other esterases are capable of performing the initial hydrolysis reaction. Likewise, considering the scarcity (if not absence, altogether) of HINTs and the presence of phosphatases in plasma, de-phosphorylation of the intermediate alanine metabolite can likely be explained by phosphatase-mediated hydrolysis. This accounts for the anionic substrate requirement for phosphatases[32]. Thus, the abundance of hydrolytic enzymes in serum explains the persistent, trans-species observation that GS-441524 is the predominant metabolite when remdesivir is administered[15,16,21]. For the fleeting duration of time that remdesivir *is* in the blood (prior to hydrolysis to GS-441524), the expression of bioactivating enzymes for McGuigan pro-drugs suggests that the highest concentrations of NTP formation deriving from remdesivir—rather than GS-441524—would occur in cell types with high expression of CES1/CTSA/HINT1-3. As described above, this largely favors the liver over the lungs (**Figure 2**).

GS-441524 is exceptionally effective and well-tolerated against clinical presentations of feline coronavirus.

Modern drug development efforts rely heavily on *in vitro* potency data before moving to *in vivo* models. This same workflow applied to the development of remdesivir when it was being explored as a possible therapy for EBOV[4,21]: when examined *in vitro*—in the absence of serum hydrolases—remdesivir exhibits markedly greater potency against EBOV-infected cell lines compared to GS-441524. Similar observations were made for cells infected with murine hepatitis virus[6]. Greater *in vitro* potency likely explains the decision for studies conducted thereafter to investigate remdesivir alone, rather than in parallel with GS-441524, *in vivo*. As a result, there are currently no studies that have compared the antiviral activities of remdesivir and GS-441524 *in vivo* (for EBOV, SARS-CoV-2, etc.). Predicated on potency differences observed *in vitro*, further *in vivo* PK and antiviral studies in NHP have focused exclusively on remdesivir[15–17,21].

Where GS-441524 has been further investigated *in vivo* is in the veterinary setting[33–35]. Cats infected with feline coronavirus (FCoV) present with a serious disease known as feline infectious peritonitis (FIP). While long considered widely fatal in its severe manifestations[36], a study conducted by Pedersen and colleagues showed that GS-441524 is capable of treating cats suffering from FIP with a 96% cure rate[34]. Pedersen noted the “impressive” safety profile of GS-441524, with no systemic signs of toxicity observed when administered subcutaneously (SC) at 4 mg/kg[34]. In a more recent study, Pedersen and colleagues escalated the dose of GS-441524 (5-10 mg/kg) to treat

neurological manifestations of FIP; for reference, this would translate to about 350-700 mg in a 70 kg human, which far exceeds the dose currently given to patients treated with remdesivir[12,18]. Even at these higher doses, they found that GS-441524 treatment resulted in the long-term resolution of neurological FIP with an excellent safety profile: minimal dose-related toxicities were observed[35].

GS-441524 shows comparable efficacy in cell-based models of human and cat cells infected with coronavirus.

In vitro potency comparisons between GS-441524 and remdesivir are ultimately moot in the context of respiratory diseases such as SARS-CoV-2, if GS-441524 is the predominant species that reaches the lungs. To better gauge the efficacy of GS-441524 against SARS-CoV-2, it may be helpful to first compare relative EC_{50} values between coronavirus infected human and cat cells, as the clinical efficacy of GS-441524 has already been well-established in cats. Prior to testing the efficacy of GS-441524 in cats *in vivo*, a report by Murphy and Pedersen examined the in vitro efficacy of GS-441524 in cell-based models of FCoV[37]. Treatment of FCoV-infected CRFK cells with GS-441524 resulted in an EC_{50} value of 0.78 μM[37] (**Figure 3a**).

In vitro comparisons between GS-441524 and remdesivir have been made in many types of human cell lines for several types of viruses[8]. Shortcomings of these experiments that preclude extrapolation to SARS-CoV-2 include: 1.) a propensity to conduct antiviral potency experiments in cancer cell lines[4,8], which have aberrant expression of various enzymes and 2.) most comparisons have not been conducted in

lung cells[21]. To the best of our knowledge, a study by Agostini and colleagues is the only one that has compared the antiviral activities of GS-441524 and remdesivir in primary human airway epithelial (HAE) cells infected with either SARS-CoV or MERS-CoV[6]. While the authors state that the mean EC_{50} value of remdesivir is lower for both SARS-CoV and MERS-CoV-infected cells, close inspection of the data reveals large standard deviations between the EC_{50} values obtained from GS-441524 and remdesivir making these potency differences not statistically significant (**Figure 3a**)[6]. Within these data, the best indicator of the utility of GS-441524 against SARS-CoV-2-infected lung cells are their data on SARS-CoV infected HAE cells, in which the reported EC_{50} for GS-441524 is 0.18 (± 0.14) μM. While comparisons across species are not straightforward, it should be noted that this EC_{50} value of 0.18 (± 0.14) μM is comparable—if not lower—than that required to exert antiviral activity against FCoV-infected cells *in vitro*. Most significantly, the EC_{50} concentration for GS-441524 against SARS-CoV-infected primary HAE cells is sustained in the plasma of NHP for nearly the entire duration of the single-dose, 24 h PK experiment conducted by Warren and colleagues (**Figure 3c**). In contrast, the EC_{50} concentration for remdesivir against SARS-CoV-infected primary HAE cells diminishes after ~2 h. The dominance of GS-441524 over remdesivir in serum was even more pronounced in Williamson's 7-day PK study, in which GS-441524 was present in serum at concentrations 1,000-fold greater than remdesivir at every measured timepoint (**Figure 3b**)[16]. Coupled with the robust antiviral activity that GS-441524 has demonstrated against FIP, these data compel further investigations into the therapeutic and prophylactic utility of GS-441524 against SARS-CoV-2 in patients.

Concluding remarks.

SARS-CoV-2 is a respiratory virus that primarily affects the lungs, with severe manifestations of Covid-19 affecting type 2 pneumocytes[27]. While remdesivir has shown some efficacy in patients with advanced Covid-19[12], its phosphate pro-drug is fundamentally not designed for lung-specific delivery. Enzymes that activate the McGuigan pro-drug are preferentially expressed in tissues such as the liver (see Human Protein Atlas), which results in uneven distribution of active NTP formation via remdesivir that disfavors the lungs. Practically, the structural complexity of the McGuigan pro-drug[38] adds unnecessary synthetic difficulty that hampers mass production—thereby impacting distribution[19]. Above all else, premature hydrolysis of the McGuigan pro-drug, followed by dephosphorylation in serum such that GS-441524 is the predominant metabolite[15,16,21] compels studies investigating its utility in patients with Covid-19. In contrast to the pro-drug activating enzymes that activate remdesivir, bioactivation of GS-441524 relies on expression of the kinase responsible for initial phosphorylation. While biochemical studies to determine the identity of this initial kinase have not been performed, the structural similarity between GS-441524 and adenosine point to adenosine kinase (ADK) as the enzyme likely responsible for this transformation. According to the Human Protein Atlas, ADK is moderately expressed across all tissues, suggesting that administration of GS-441524 would result in even distribution across tissues. The remarkable safety profile of GS-441524—indicated by selectivity indices *in vitro* (EC_{50}/CC_{50} ratio)[4,8,11] and by clinical observations in cats[33–35]—suggest that higher dosing could be achieved with GS-441524 compared to remdesivir without encountering serious adverse effects. This would enable higher NTP loading in

the lungs. GS-441524 is also a structurally simple molecule that is easier to synthesize compared to remdesivir[4], which would ease mass production and distribution. Its low molecular weight and hydrophilicity would also make formulation into an inhalable treatment much simpler; for instance, aerosolization of the nucleoside analogue, ribavirin, only requires sterile water and sodium chloride[39]. Inhalable delivery is not only attractive for direct administration to the lungs but would also enable prophylactic delivery outside of a hospital setting. Such applications are particularly attractive, given the strong benefits of prophylactic antiviral treatment[17] evidenced in other viral settings (e.g.: PrEP for HIV)[40,41]. Taking these advantageous properties into account—amidst the documented premature serum hydrolysis of remdesivir to GS-441524[15,16,21]—we see several advantages to using GS-441524 over remdesivir for patients with Covid-19. Further investigations into its anti-Covid-19 are thus imperative.

Author contributions.

V.C.Y. and F.L.M. researched and analyzed the literature. V.C.Y. wrote the manuscript.

Author disclosures.

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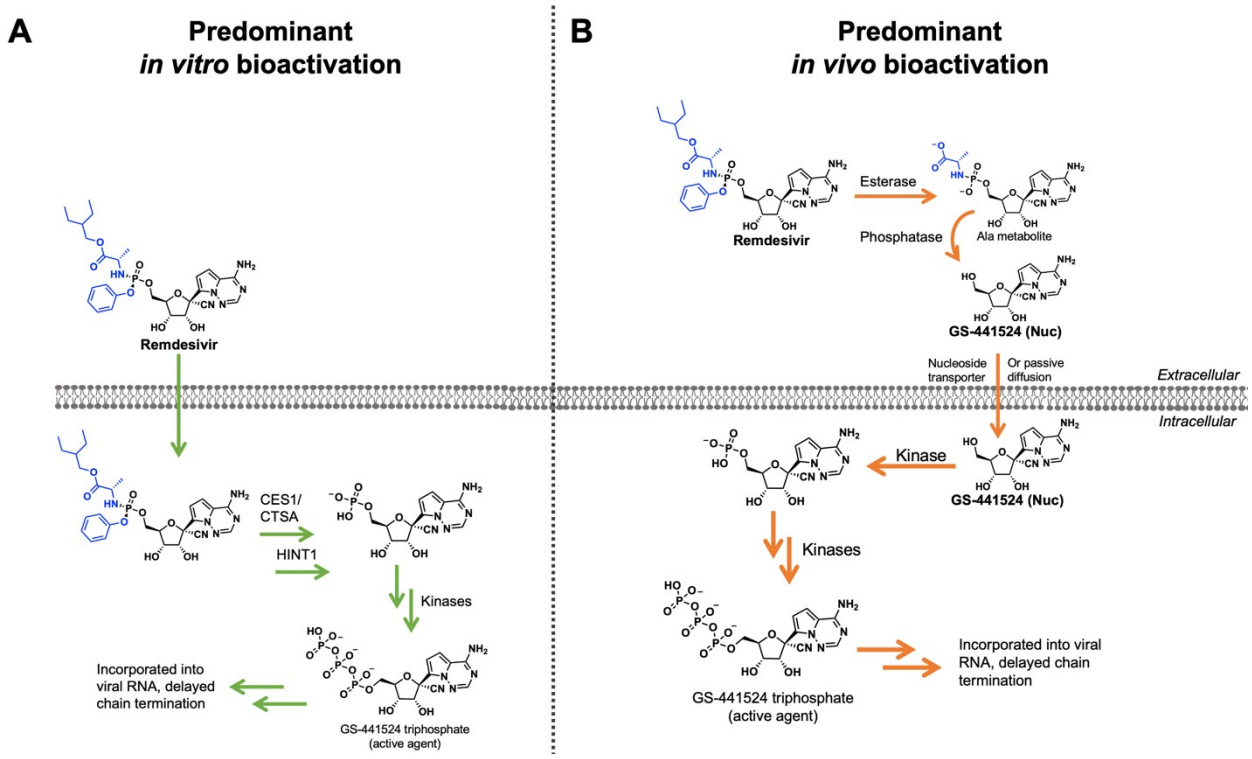

Figure 1. McGuigan pro-drugs on remdesivir are prematurely hydrolyzed in serum. (**A)** The ideal bioactivation of remdesivir predominately occurs *in vitro*. (**B)** The presence of serum enzymes *in vivo* predominately results in premature hydrolysis of the phosphate pro-drugs, followed by dephosphorylation to the nucleoside, GS-441524.

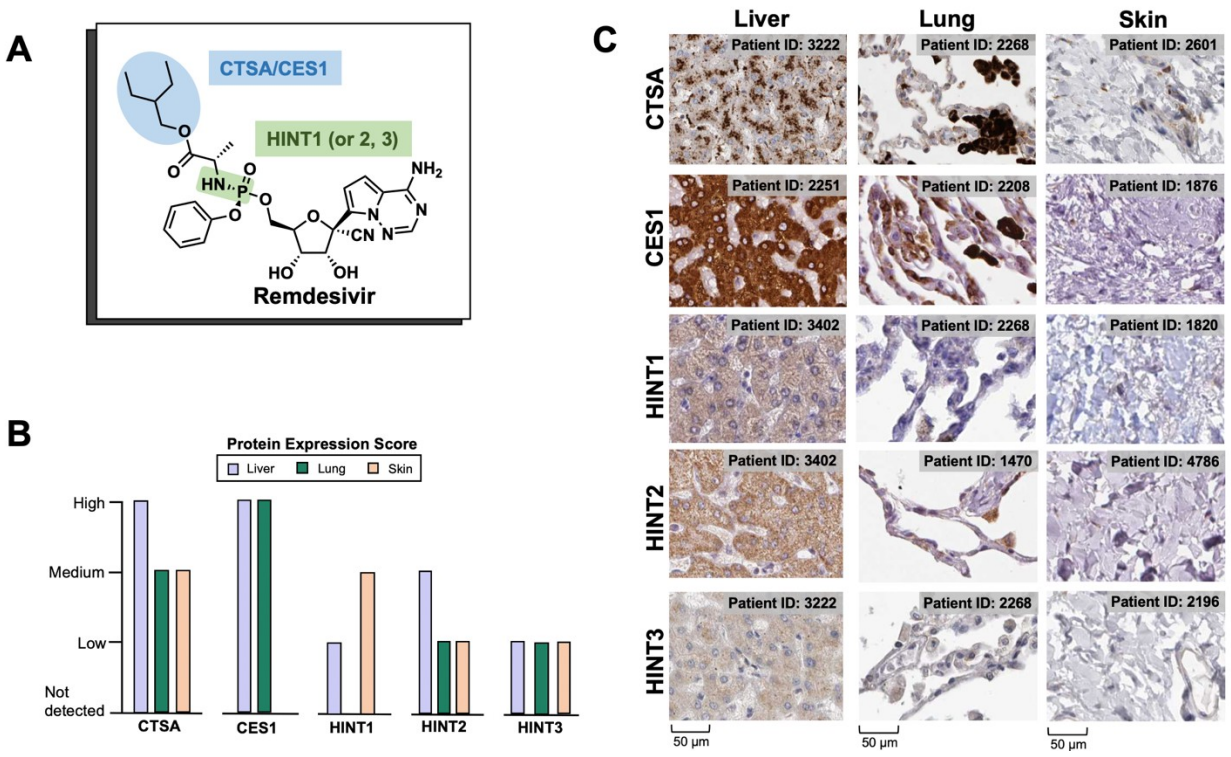

Figure 2. McGuigan pro-drugs on remdesivir are preferentially bioactivated in the liver. **(A)** Labile pro-drug moieties on remdesivir with corresponding bioactivation enzymes. **(B)** Protein expression scores adapted from the Human Protein Atlas. Overall, ProTide bioactivating enzymes are more highly expressed in the liver than in the lungs. For HINT1, protein expression was not detected in lung. **(C)** Immunohistochemistry (IHC) images from the Human Protein Atlas indicating expression for ProTide bioactivating enzymes. Brown regions indicate enzyme expression while blue regions indicate absent expression. For the lung, pneumocytes—cells frequently infected by Covid-19—are characterized by a threadlike appearance. Expression in the liver is generally higher compared to lung for all enzymes. For CTSA, darkly stained regions are associated with macrophages. IHC images for the skin are included to show lack of enzyme expression. Antibodies used: CTSA (CAB024930), CES1 (HPA046717), HINT1 (HPA044577), HINT2 (HPA059109), HINT3 (HPA027914).

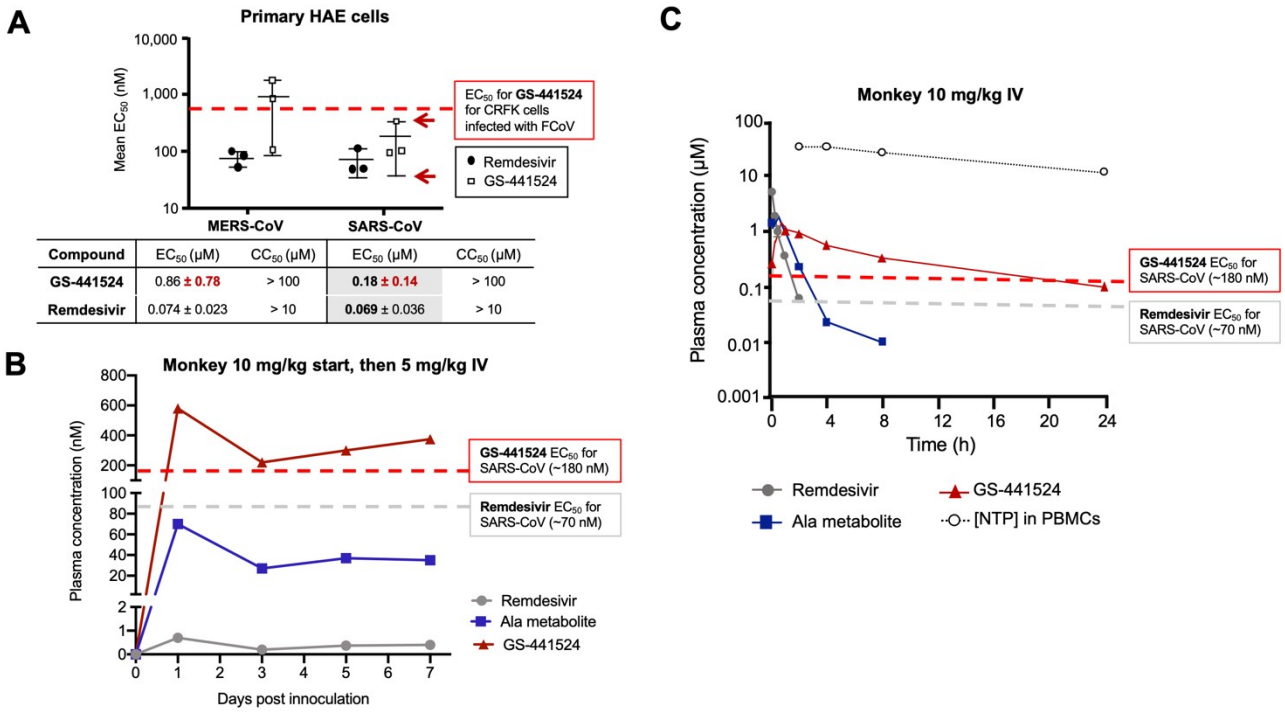

Figure 3. Unlike remdesivir, GS-441524 persists in serum at concentrations above the EC_{50} value required against SARS-CoV-infected primary HAE cells for long durations. (A) *In vitro* potency data re-plotted from Agostini et al. *mBio*, 2018[2]. Primary HAE cells were infected with either MERS-CoV or SARS-CoV and treated with either GS-441524 (open squares) or remdesivir (closed circles). Mean EC_{50} of GS-441524 for SARS-CoV-infected HAE cells was found to be 0.18 ± 0.14 μM (note large standard deviations, red arrows). A study by Murphy et al. shows that GS-441524 has an EC_{50} value of 0.78 μM against FCoV-infected CRFK cells (red dashed line)[33], which is higher than the EC_{50} value for GS-441524 against SARS-CoV-infected primary HAE cells. **(B)** Estimated metabolite concentrations for a PK experiment in a SARS-CoV-2 primate model replotted from Williamson et al. *bioRxiv*, 2020[16]. Primates were initially injected IV with 10 mg/kg of remdesivir 12 h post-inoculation with SARS-CoV-2 and then 5 mg/kg of remdesivir every 24 h after. Throughout the experiment, GS-441524 is present in

serum at concentrations ~1000-fold higher than remdesivir; the concentration of GS-441524 is consistently above the EC_{50} value in SARS-CoV-infected primary HAE cells (red dashed line) at all timepoints taken in the experiment. In contrast, the concentration of remdesivir in serum never exceeds that required to give the EC_{50} value against SARS-CoV-infected primary HAE cells (grey dashed line). **(C)** PK data replotted from Warren et al. *Nature*, 2016[3] following IV injection of 10 mg/kg of remdesivir in NHP. Dashed lines indicate the approximate EC_{50} values of GS-441524 (red) or remdesivir (grey) needed for antiviral activity in SARS-CoV primary HAE cells obtained in (A). Unlike remdesivir, the concentration of drug required to give the EC_{50} value against SARS-CoV primary HAE cells is maintained for significantly longer with GS-441524 than with remdesivir.

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