Why Remdesivir Failed: Preclinical Assumptions Overestimate the Clinical Efficacy of Remdesivir for COVID-19 and Ebola

Victoria C. Yan1* and Florian L. Muller2
1 Copycat Sciences, Boston, MA, USA
2 Sporos Bioventures, Houston, TX, USA
*Email: victoria.yan@copycatsciences.com Twitter: @victoriacyanide (V.C.Y.)

Abstract
Remdesivir is a nucleoside monophosphoramidate prodrug that has been FDA-approved for COVID-19. However, the clinical efficacy of remdesivir for COVID-19 remains contentious, as several trials have not found statistically significant differences in either time to clinical improvement or mortality between remdesivir-treated and control groups. Similarly, the inability for remdesivir to provide a clinically significant benefit above other investigational agents in patients with Ebola contrasts with strong, curative preclinical data generated in rhesus macaque models. For both COVID-19 and Ebola, significant discordance between the robust preclinical data and remdesivir’s lackluster clinical performance have left many puzzled. Here, we critically evaluate the assumptions of the models underlying remdesivir’s promising preclinical data and show that such assumptions over-predict efficacy and minimize toxicity of remdesivir in humans. Had the limitations of in vitro drug efficacy testing and species differences in
drug metabolism been considered, the underwhelming clinical performance of remdesivir for both COVID-19 and Ebola would have been fully anticipated.

Introduction

Remdesivir (RDV) is a nucleoside monophosphoramidate prodrug that has been FDA-approved for COVID-19 principally on the basis of one double-blind, randomized control trial (RCT) (1), which demonstrated a faster median time to recovery in RDV-treated patients by about 5 days (10 days) compared to the placebo group (15 days). However, the clinical efficacy of RDV remains contentious, as a smaller double-blind RCT showed no statistical difference in clinical improvement between RDV- and placebo-treated patients (2); interim results from a larger RCT conducted by the WHO has found no significant difference in mortality between patients treated with RDV compared to those treated with the standard-of-care (SOC) (3, 4), prompting the organization to recommend against using RDV for COVID-19 (4). Such clinical results diametrically oppose the impressive preclinical data, which showcased RDV’s strong, broad-spectrum antiviral activity in cell culture (5–7) and in preclinical species (8–11). Here, we show that overestimations of RDV’s clinical performance arise from several preclinical assumptions made at both the in vitro and in vivo levels, with the principal assumption being that the conventional drug development framework is relevant to prodrugs such as RDV. We show that this preconceived notion fails to account for the significant differences between “soft” drugs (those that are subject to metabolic transformation) such as RDV and “hard” drugs (those that are metabolically inert) which permeate the FDA-approved drug landscape (12). By re-assessing key data on RDV at
the cell culture and preclinical organismal levels, we elucidate the predictability of RDV’s clinical underwhelming performance and provide insights for future development of phosphate prodrugs.

Remdesivir is a McGuigan prodrug that predominantly deposits bioactive triphosphate in the liver

RDV is a phosphoramidate prodrug of the McGuigan (ProTide®) class that was originally designed to deliver a membrane-impermeable nucleoside triphosphate (NTP) analogue (GS-443902) for the treatment of hepatitis C (HCV) (13–16). McGuigan prodrugs such as RDV, which contain a phosphate protected by an aryloxy and amino acid ester moieties, are intended for intracellular conversion of the active NTP through bioactivation by carboxylesterase 1 (CES1), cathepsin A (CTSA), and histidine triad nucleotide binding protein 1 (HINT1) (16, 17). During RDV’s development, several other nucleoside and nucleotide analogues were also being investigated for their anti-HCV efficacy, with the most advanced compounds being pyrimidine analogues (NCT00120835, NCT00869661, NCT01096576). As purine analogues, RDV and its sibling compounds (14) were thus developed against the backdrop of pyrimidine analogues. Application of the McGuigan prodrug strategy onto anti-HCV pyrimidine analogues sought two aims: 1.) rapid loading of the active NTP in the liver, where HCV is trophic and 2.) overcoming the rate-limiting initial phosphorylation step observed for some pyrimidine analogues (18). While the former reason is generally applicable to multiple phosphate prodrugs, the latter reasoning has yet to be explicitly demonstrated for purine (adenosine) analogues such as RDV and its parent nucleoside, GS-441524.
In fact, the prohibitively slow initial phosphorylation step appears to be limited to some uridine analogues, such as RO2433 (parent nucleoside of sofosbuvir) (18), with many cytidine and thymidine analogues appearing to undergo initial phosphorylation quite readily (19, 20). Multiple studies have already demonstrated the ability for GS-441524 to undergo conversion to the active NTP through direct measurement of GS-443902 levels or through indirect assessment of the low micromolar EC\textsubscript{50} value of GS-441524 against virus-infected cells; varying EC\textsubscript{50} values for GS-441524 and RDV in head-to-head comparisons further suggest the cellular potency of either compound is cell-line and cell-type dependent (Supplementary File 1) (5, 7, 21–23) despite the fact that the exquisite specificity of GS-443902 for the SARS-CoV-2 RdRp is undisputed (K\textsubscript{m} ~9 nM) (24–26). Thus, as an artifact of its initial HCV indication, the primary function of the McGuigan promoiety on RDV is preferential hepatic bioactivation (27).

Much of the initial excitement around the potency of RDV in cell culture models of SARS-CoV-2 centered on its low micromolar potency in a variety of cell lines and cell types, including primary human airway epithelial (HAE) cells, in which the lowest reported EC\textsubscript{50} value for RDV against SARS-CoV-2 was obtained (5). Against Ebola virus (EBOV)-infected cells in culture, treatment with RDV yielded similarly impressive, nanomolar EC\textsubscript{50} values (6, 21, 28) (Supplementary File 1). Among other oversights discussed subsequently, these superficially impressive \textit{in vitro} efficacy data were assessed in isolation—without considering how such data compare to RDV’s cytotoxicity in hepatocytes. Placed in context of primary human hepatocytes (PHH) \textit{in vitro}, the levels of active NTP formed by primary HAE cells are unambiguously
dominated by those formed in PHH (Figure 1). Simply put: even in the cell model where RDV performs best against SARS-CoV-2, levels of active NTP are still approximately 4-fold higher in PHH than in primary HAE cells when both cell types are subject to identical culturing and treatment conditions (5, 29).

Conventional cell culture protocols fail to account for the complex pharmacokinetics of remdesivir in vivo

Discrepancies between the *in vitro* and *in vivo* conditions are exacerbated for prodrugs that are susceptible to extracellular metabolism such as RDV. Key considerations that are particularly pertinent for (phosphate) prodrugs but tend to be overlooked under standard cell culturing protocols include magnitude of exposure, exposure time, and distribution patterns incurred by route of administration. One could argue that such shortcomings could be accounted for in subsequent *in vivo* evaluations. Nevertheless, it appears that decisions to advance or halt the development of certain prodrugs are contingent upon *in vitro* and *ex vivo* data without considering the assumptions inherent in standard cell culture protocols or without revision to the way in which cell culture assessments are performed even after observing the prodrug’s pharmacokinetic behavior *in vivo*. For instance, a typical cell-based screening assay involves incubating cells with virus for approximately 30-60 minutes before removing virus-containing media, washing the cells, and then incubating the cells with drug for 48-72 hours (5, 6, 30).

While appropriate for drugs with long T<sub>1/2</sub>s (i.e., most “hard” drugs), such conditions skew efficacy results for “soft” drugs with short T<sub>1/2</sub>s such as RDV. Under standard screening conditions, 4 assumptions are being made (Table 1): 1.) viral load is constant,
2.) the target population of cells is constantly exposed to drug for 48-72 hours at supraphysiological concentrations of drug, 3.) the ratio of drug concentration to the number of target cells is high, 4.) preferential extraction by other organs or cell types is negligible. Using RDV as an example, such assumptions are inconsistent with its clinical pharmacokinetics, given that it has a $T_{1/2}$ of less than 1 hour in humans (31, 32). For the brief time that intact RDV is present in systemic circulation, it is both unclear and unlikely that tissue distribution is uniform, despite RDV’s large $V_z$ (33); preferential hepatic extraction (Figure 1) and competing plasma esterase hydrolysis undermines the ability for RDV to durably reach the primary site of SARS-CoV-2 infection (type II alveolar cells) and exert its antiviral activity (31, 32).

A set of experiments by Wang and colleagues demonstrated the magnitude of SARS-CoV-2 inhibition by RDV in Vero E6 cells was largely contingent upon the duration of drug exposure (30). Seeking to probe the mechanism of SARS-CoV-2 inhibition by RDV and chloroquine (CQ), Wang et al. performed a series of “prophylactic,” “entry” and “post entry” experiments (Figure 2a, b). Of the 3 conditions tested, the “entry” experiment most closely resembled the in vivo pharmacokinetics of RDV, as cells were pulsed with drug for just 3 hours before being washed and allowed to incubate with fresh media for the duration of the experiment. Like RDV, CQ is subject to metabolism to its main metabolite, N-desethylchloroquine, (DCQ). Though unlike RDV with its short $T_{1/2} < 1$, the $T_{1/2}$ of CQ is approximately 18-30 days (34, 35). Thus, the short pulsing experiment more accurately reflects the in vivo pharmacokinetic situation for RDV but not CQ. Noting differences in the tested concentrations of RDV and CQ, RDV
demonstrated no inhibition of SARS-CoV-2 after a 3 h drug pulse at 3.7 μM; in contrast, CQ maintained similar levels of SARS-CoV-2 inhibition across all 3 conditions (Figure 2a “entry’’). Discordant inhibitory values by RDV across the 3 conditions, suggest that the antiviral efficacy of RDV is considerably diminished when target cells are pulsed—rather than continuously exposed—to RDV. One potential confounder to this interpretation could be that these experiments were performed in Vero E6 cells, which have rather high expression of p-glycoprotein (p-gp; ABCB1, Figure 2c). It has been reported that RDV is a substrate for ABCB1 (33); in contrast, CQ has been shown to be a substrate for ABCC1 (36), which is expressed at lower levels in Vero E6 compared to ABCB1 (Figure 2c). Thus, while pulsed experiments in Vero E6 may exaggerate the reduced anti-SARS-CoV-2 activity of RDV, the totality of these data points to the dependency of RDV’s antiviral activity in the target cell population on duration of exposure. In a more recent publication, Mackman and Cihlar et al. compared the formation of GS-443902 when normal human bronchial epithelial (NHBE) cells were incubated with either pulsed (2 h) or continuous (48 h) concentrations (1 μM) of drug in an attempt to better recapitulate the transient in vivo T₁/₂ of RDV (Figure 2d) (37). Expectedly, concentrations of GS-443902 were considerably diminished under pulsed conditions (Figure 2d), which supports reduced antiviral activity by RDV observed by Wang et al. under “entry” conditions (Figure 2a). In fact, when the levels of GS-443902 generated by RDV under pulsed and continuous conditions are compared to the C₂₄ of GS-443902 corresponding to an EC₅₀ of 10 nM in SARS-CoV-2-infected HAE cells (5), it becomes apparent that short exposure to RDV is insufficient to achieve the desired,
low nanomolar antiviral effects *in vitro* (Figure 2d). This supports the dramatically reduced antiviral activity observed by Wang et al. in Vero E6 cells (30).

While the aforementioned experiments address the second assumption (drug exposure), they do not resolve the third (ratio of drug concentration to cell number) and fourth (preferential extraction by other organs) assumptions and thus must be taken with grains of salt rather than as definitive extrapolations of *in vivo* behavior (Table 1). The pulsed experiments described by Wang and Mackman and Cihlar, assume the ratio of drug concentration to cell number is high (Table 1, Assumption 3), which opposes the *in vivo* reality where the ratio of drug concentration to cell number is low and is exacerbated by uneven drug distribution due to preferential metabolism in cell types such as the liver (Table 1, Assumption 4) (27). It is admittedly difficult to account for these *in vivo* factors in cell culture; however, the challenge of modeling them does not give license to disregard their significance. Indeed, Mackman and Cihlar et al. demonstrated that a 10 mg/kg single IV dose of RDV in African green monkeys and cynomolgus macaques resulted in high accumulation of GS-443902 and RDV metabolites in the liver and kidney, suggesting non-uniform distribution of the prodrug (Table 1, Assumption 4; Supplementary Figure S1) and re-emphasizing the inability for conventional cell culture protocols to account for this important *in vivo* reality (37). As is apparent in the stark contrast between the *in vitro* potency of RDV and its modest clinical efficacy (2, 3, 28, 38), results from *in vitro* experiments offer an incomplete portrayal of the *in vivo* situation; data obtained from such studies should thus be
reconsidered in light of the 4 key assumptions (Table 1) when making judgements on
which compounds to advance to the clinic.

Prolonged treatment with remdesivir in non-human primates fails to anticipate
clinically significant hepatotoxicity

While biochemical and immunohistological data suggest that preferential hepatic
metabolism of RDV could result in liver related dose limiting toxicities (DLTs) in the
clinic, such shortcomings were not accounted for in non-human primates (NHPs)
subjected to repeated dosing of RDV. During a 28-day EBOV challenge study, rhesus
macaques were subject to various dosing regimens of RDV for 12 days (8). There were
two particularly outstanding findings from this study. First, only the continuous 10 mg/kg
dose resulted in “profound suppression of EBOV replication and protected 100% of
EBOV-infected animals against lethal disease, ameliorating clinical disease signs and
pathophysiological markers, even when treatments were initiated three days after virus
exposure when systemic viral RNA was detected in two out of six treated animals.”
Second, 12-day treatment with RDV at 10 mg/kg did not lead to significant elevations in
ALT/AST (Figure 3b). The latter is particularly surprising, as a 10 mg/kg dose in a 50-
70 kg human directly equates to 500-700 mg of RDV, which is more than 2-times higher
than the amount of RDV used clinically and more than twice the duration of RDV
currently recommended for COVID-19 (1–3, 39, 40). Even with allometric scaling (41),
10 mg/kg RDV in NHP translates to about 168-284 mg of RDV in humans (Figure 3c),
which exceeds the recommended dose of 200 mg loading followed by 100 mg for 5-10
days. Unlike NHP, which can tolerate 10 mg/kg RDV for 12 days without significant
transaminase elevations, humans would not be able to withstand the allometrically scaled dose for the same duration, given that duration-dependent, low grade transaminase elevations had already emerged in 25% and 75% of healthy human volunteers administered 150 mg QD for 7 and 14 days, respectively (Figure 3a versus b) (31, 42). Allusions to higher hepatic extraction of McGuigan prodrugs in human compared to monkey hepatocytes had been demonstrated in another Gilead study with sofosbuvir (SOF), another McGuigan prodrug (43). Following a 2-hour pulse with 10 μM SOF in primary hepatocytes across species, human hepatocytes formed about 3-fold higher active SOF NTP compared to monkey hepatocytes (Figure 3d). Likewise, \( C_{\text{max}} \) concentrations of SOF NTP in vivo was found to be much higher in human liver (~50 μM) (44) compared to in monkey liver (~5 μM) when SOF was administered at the allometrically scaled dose (400 mg in humans; Figure 3d) (43). Coupled with the observation that 12-day dosing with RDV at 10 mg/kg does not yield significant ALT/AST elevations in NHP (8), these data strongly suggest there is preferential hepatic extraction of McGuigan prodrugs in humans compared to NHP.

As a result of model-specific disparities in the hepatic extraction of McGuigan prodrugs, the dose-escalation that would likely be required to clarify RDV’s antiviral activity in COVID-19 (2) and Ebola (45, 46) patients is prohibited due to on-target hepatoxicity (31). Thus far, the largest clinical study that examined the relationship between RDV treatment and reduction of SARS-CoV-2 titers was published by Wang and colleagues in the Lancet (2). While criticized for its small trial size (N=237), this study showed that RDV was unable to demonstrate: 1.) a statistically significant time to improvement
compared to placebo controls and: 2.) reduction in viral loads in the upper and lower airways compared to controls (2). Since the Wang study was published, some unique case reports have described the ability for RDV to reduce viral loads in the upper airway (47, 48). Still, the bulk of available data suggest that the current recommended dose (200 mg loading, 100 mg maintenance for 5-10 days (49)) is insufficient (42) to yield the robust antiviral activity to which many *in vitro* studies have alluded (5, 38, 50).

Insufficient dosing in humans is corroborated by data on RDV in EBOV-infected patients, in which RDV was unable to demonstrate significant reductions in viral loads at the standard dose (200 mg loading, 100 mg maintenance for 9-12 days) in hospitalized patients (46) or at a lower dose (100 mg for 5 days) while on treatment in patients with viral persistence in semen (45). Preclinical evaluation of RDV at the allometrically scaled dose (10 mg/kg loading, 5 mg/kg maintenance for 6 dpi) in rhesus macaque models with mild COVID-19 (11, 51) found there was no significant reduction in viral titers in the upper airway by RDV compared to untreated animals—with the exception of the trachea (Figure 4a, b) at 7 dpi. Treatment with RDV yielded a global reduction in viral load in all examined lobes of the lung; however, this dose was unable to exert a more profound reduction in viral titers during the treatment period (Figure 4a). The underwhelming nature of these data are underscored by the close relationship between viral load and disease severity (52). Likewise, the relationship between RDV dose and degree of viral reduction is readily observed in a rigorous study designed to investigate the optimal RDV dose for the treatment of EBOV in non-human primates (NHP). Among the 12-day dosing regimens investigated in EBOV-challenged NHP, two schedules initiated at 3 dpi yielded 100% survival rate at 28 dpi: 1.) 10 mg/kg and 2.) 10 mg/kg
loading followed by 3 mg/kg maintenance for 11 days. Despite yielding the same survival efficacy, the authors note that “antiviral effects were consistently greater in animals administered repeated 10 mg/kg GS-5734 [RDV] doses (8)” (Figure 4d). In fact, the direct relationship between drug dose, reduction in antiviral activity, and improved disease outcomes is most apparent when comparing the outcomes between the lowest (3 mg/kg beginning at 2 dpi) and highest (10 mg/kg beginning at 3 dpi) dosing cohorts tested (Figure 4d light purple vs. light blue): whereas the former yielded 66% survival by 28 dpi and moderate reductions in viral titers early in treatment, the latter yielded 100% survival by 28 dpi and robust reductions in viral titers early in treatment. In contrast to the large, unexplained disparities in survival for the 10/3 mg/kg dosing regimen (Figure 4e, dark purple vs. light purple), frank reductions in viral titers and 100% survival were unique to the 10 mg/kg D3 cohort (Figure 4d, e, light blue). Viewed totality, these data demonstrate that the antiviral effects of RDV can be augmented by up-dosing. As RDV had already been investigated for EBOV before SARS-CoV-2, knowledge of its safety and tolerability in healthy human volunteers had already been established (31) well before preclinical studies into its in vivo efficacy against SARS-CoV-2 had begun, 10 mg/kg/day dosing was not evaluated (11). However, the dose-dependent decreases in viral titers with EBOV (8), coupled with the higher affinity of GS-443902 for the SARS-CoV-2 RdRp compared to the EBOV RdRp (24), strongly suggest that 10 mg/kg/day dosing would have demonstrated a more obvious antiviral benefit in NHP models. Inadequate dosing of RDV in humans likely explains suboptimal antiviral effects for both COVID-19 and EBOV. Though Warren et al. concluded that 10 mg/kg/day dosing
offered the best response in EBOV-challenged NHP (8), liver-related DLTs in healthy human volunteers (Figure 3) ultimately precluded administration at the human equivalent dose (HED) for a similar duration required for more dramatic reductions in viral titers in NHP (31). If the 10 mg/kg RDV dose cohort had been directly translated in humans, an HED of 168-284 mg RDV for 12 days would have been administered. Instead, RDV was instead administered at 200 mg on D1, followed by 100 mg from D2 and continuing to D9-13 for EBOV or D2-10 for COVID-19 (1, 2, 46). These dosing schedules resemble the 10/3 m/kg schedule used for NHP challenged with EBOV or the 10/5 mg/kg schedule used for NHP challenged with SARS-CoV-2—both of which did not result in stark reductions in viral titers for their respective virus (Figure 4b, d) (11, 21). Viewed together, these data suggest that reductions in viral titers—and the closely related improvements in disease outcomes (Figure 4d, e)—are dose-dependent. Stronger antiviral effects by RDV would likely be observed in humans if it were possible to safely escalate the dose (42, 53). Considering the dose recommendations for RDV, large-scale studies on the relationship between up-dosing RDV and reductions in viral titers have thus not been conducted. However, there exists a single notable case report from 2015 documenting the effects of high-dose RDV in reducing EBOV titers in a Scottish nurse who relapsed with EBOV—the first time RDV had been administered in an EBOV patient (53). While she had received other agents during her hospitalization, there was a period in which only RDV was administered on a compassionate use basis—before safety in humans had been fully characterized. This patient received a daily IV infusion of 150 mg RDV for 3 days, which was then increased to 225 mg for 11 days. In addition to being the highest reported dosing schedule for any human, it most
closely resembles the 12-day 10 mg/kg dosing schedule that was found to be most
effective in the rhesus model of EBOV (8). While the report indicates that the first
reported decline in viral RNA occurred 1 day before RDV treatment began, it is
interesting to note that a sharp, sustained reduction in viral titers in CSF and plasma
occurred during RDV treatment. Ultimately, clinical recovery was observed when RDV
was supplemented with dexamethasone on day 4 of 14 of RDV treatment (53).
Cognizant of the inherent limitations of a case report, these data hint that up-dosing
could clarify the magnitude of antiviral activity by RDV and could warrant further
investigation into the dose-dependent nature of its antiviral activity in humans. Due to
the low grade transaminase elevations observed in healthy human volunteers treated
with RDV (31), constraints on the RDV dose have understandably prevented larger
scale studies from being initiated to support initial observations made in the Scottish
nurse case report (53). Both *in vitro* and phase 1 data indicate that the up-dosing that
would be required to boost the presently questionable antiviral effects of RDV (2, 3) is
precluded by its nephro- and hepatotoxicity, (29, 31). Against liver-derived cell lines and
PHH *in vitro* (**Figure 1b**), RDV demonstrated uniquely low CC$_{50}$ values (2.5-6 µM)
compared to >100 µM for its parent nucleoside, GS-441524, which demonstrates similar
anti-SARS-CoV-2 activity (29); this concurs with its putative mechanism of bioactivation
by enzymes that are highly expressed in the liver (16). These *in vitro* data explain the
dose-duration-dependent emergence of low grade transaminitis observed in the phase
1 dose escalation study in healthy human volunteers: administration of RDV at 150 mg
for 7 or 14 days resulted in 25% and 75% of volunteers experiencing grade 1/2
ALT/AST elevations, respectively (**Figure 3a**) (31). Likewise, the *in vitro* CC$_{50}$ value
against renal proximal tubule epithelial cells (RPTECs)—while not as low as that observed in liver cell lines—is considerably lower (12.9 µM) than that observed for its parent nucleoside (>100 µM; Supplementary Figure S1) (29). Coupled with the documented renal elimination of the solubilizing excipient, Captisol® (54), the lower CC50 value by RDV observed in kidney cells in vitro could partly explain observations of exacerbated kidney injury in patients with compromised renal function treated with RDV (42, 55). The latter explanation concurs with tissue distribution studies of the total nucleoside (GS-441524) and nucleotide metabolites (mono and di-phosphates of GS-441524 and the triphosphate, GS-443902) in NHPs following 10 mg/kg single dose IV administration of RDV formulated with the same Captisol®-containing solution used in the clinic (37). Compared to NHP administered 20 mg/kg IV GS-441524 formulated without Captisol®, a significantly higher proportion of total nucleoside and nucleotide metabolites were observed in the kidneys of NHPs dosed with RDV (Figure X) (37).

Thus, the unique sensitivity of liver and kidney cells to RDV due to its identity as a hydrophobic McGuigan prodrug (29)—reinforced by the liver and kidney-related exclusion criteria in its clinical trials (1–3)—indicates that the up-dosing that would be required to boost RDV’s antiviral effects would be likely and broadly result in concomitant hepatic- and nephrotoxicity.

**Conclusion**

Preclinical evaluation of compounds should be dynamic. Especially in the case of prodrugs with short in vivo T1/2s such as RDV, cell culture protocols should be revised upon receipt of in vivo data to better reflect the prodrug’s pharmacokinetics in vivo.
Standard workflows appear to over-emphasize low EC$_{50}$ values while underappreciating the contributions of drug exposure, tissue-specific localization, and a drug's CC$_{50}$ value. While it is true that exigency of the COVID-19 pandemic made RDV amenable to rapid investigation into its utility as a therapeutic, its underwhelming clinical performance ought to have incited a meticulous investigation to identify reasons for RDV's discording preclinical strength and clinical weakness. Assumptions inherent in cell-based screening protocols—while perhaps adequate for hard drugs—are poorly suitable for esterase-labile prodrugs such as RDV, in which transient exposure to the target cell population is exacerbated by uneven tissue metabolism. At the in vivo level, an over-reliance on NHP models when studying McGuigan prodrugs fails to anticipate RDV's hepatotoxicity in humans at dosing intervals that are well-tolerated in NHP—presumably due to species differences in hepatic extraction of McGuigan prodrugs. Based on studies spearheaded by Wang and Murakami with SOF, it appears the efficiency of McGuigan prodrug metabolism in human hepatocytes is greater than that of NHP but less than that of PXB mice and dogs. This would suggest that the contribution of dog models for safety and tolerability should not be underappreciated in favor of NHP models when considering the in vivo behavior of McGuigan prodrugs such as RDV. Taken together and stated simply: conventional approaches to in vitro and in vivo studies for hard drugs are poorly suited to complex prodrugs like RDV. If the logic and interpretation of RDV preclinical data were correct, then a more distinct clinical benefit would be anticipated. This is not the case. The gap between the strong preclinical data with RDV across many virus models and its questionable
clinical activity (2, 3) suggests that there are assumptions being made at the preclinical level that do not reflect the conditions observed in patients. In light of RDV’s clinical performance, we have attempted to explain the causes for such a discordance by parsing the nuances of typical preclinical models used to judge RDV’s efficacy. Our careful analysis offers insight that should be considered when selecting (phosphate) prodrugs for clinical advancement.

**Transparency Declaration**

V.C.Y. is the CEO of Copycat Sciences, a company developing antiviral nucleoside analogues.

**Author Contributions**

V.C.Y. performed research, analysis, and wrote the manuscript. F.L.M. provided critical comments and assisted in the preparation of the manuscript.

**Author Bios**

Victoria Yan, M.S. received her undergraduate degree in biochemistry from Mount Holyoke College in 2018 and her M.S. in therapeutics & pharmacology from the University of Texas Health Science Center at Houston/MD Anderson Cancer Center in 2020. As a masters’ student in the lab of Florian Muller, she developed novel methods for the synthesis of phosphoramidate prodrugs and synthesized several prodrugs of a phosphonate-containing inhibitor of enolase 2 for the treatment of cancers harboring deletions of enolase 1. During COVID-19, she directed her knowledge of phosph(on)ate prodrug metabolism to remdesivir and has been advocating for the use of its parent
nucleoside, GS-441524, for COVID-19 treatment. Yan is the first to report the safety, tolerability, and pharmacokinetics of orally administered GS-441524 in woman. As an extension of her graduate work, she founded Copycat Sciences, a phosph(on)ate prodrug company, with foci in virology, precision oncology, and inborn errors of metabolism.

Florian Muller, Ph.D. received his undergraduate degree from Washington State University and his Ph.D. from the University of Texas Health Science Center at San Antonio in 2007. His undergraduate and graduate careers were dedicated to the study of mitochondrial reactive oxygen species in aging and neurodegenerative disease. During his postdoctoral training, Muller joined the lab of Ron DePinho, where he applied his training in basic biochemistry to precision oncology, demonstrating the utility of passenger genomic deletions of metabolic enzymes, as pharmacologically targetable vulnerabilities in cancer. As an Assistant Professor at M.D. Anderson Cancer Center, brought this concept closer to the clinic, by synthesizing small molecules exploiting such vulnerabilities. This led to the development of novel phosphonate prodrugs, the knowledge of which became relevant during COVID-19 given remdesivir’s identity as a phosphate prodrug. Muller is presently a Sr. Director Sporos Bioventures, a venture capital firm specializing in early oncology start-ups.
Figure 1. Remdesivir is preferentially bioactivated in hepatocytes. (A) RDV NTP (GS-443902) formation in PHH and HEp-2 cells is about 4-fold higher than in HAE cells and about 12-fold higher in PC-3 cells than in HAE cells. Mean of data replotted from Pruijssers et al. Cell Rep. (2020; HAE (5)) and Xu et al. Antimicrob. Agents Chemother. (2020; PHH, HEp-2, PC-3 (29)). Cells were seeded at approximately $1 \times 10^6$ cells/well and were incubated with 1 μM RDV for 24 h. Levels of NTP in HAE cells represent the average of quadruplicate technical replicates from 2 donors. (B) Comparison of SIs for CC$_{50}$ values of RDV in HEp-2, HepG2, PC-3, and PHH and RDV EC$_{50}$ values obtained
in A549, Calu3, HAE, and VeroE6 cells infected with SARS-CoV-2. EC$_{50}$ values used were obtained from Xie et al. Nat. Comm. (2020; A549 (38)) and from Pruijssers et al. Cell Rep. (2020; Calu3, HAE, Vero E6) (5).

Table 1. Assumptions in standard cell culture procedures do not reflect the in vivo behavior for prodrugs like RDV

Figure 2. Duration of drug exposure impacts the antiviral efficacy of remdesivir. (A) Continuous exposure to RDV leads to durable in vitro inhibition of SARS-CoV-2 but pulsed treatment with RDV results in diminished antiviral activity in Vero E6 cells. Mean of triplicate data re-plotted from Wang et al. Cell Res. (2020) (30). Across the three experiments, RDV was tested at 3.7 µM and CQ was tested at 10 µM. Due to its long in vivo $T_{1/2}$, CQ serves as a positive control. (B) Corresponding treatment procedures for “prophylactic”, “entry”, and “post-entry” experiments described in (A). Pulsed experiments correspond to the “entry” trial. (C) RNA-seq data showing expression of relevant prodrug bioactivating enzymes for RDV in Vero E6 cells represented as the mean ± SD of 3 experiments (Supporting Information). ADK = adenosine kinase; AK2 = adenylate kinase 2. AK2 and SLC29A3 were found to mediate RDV potency and toxicity in a genome-wide CRISPR screen (56). (D) Formation of active triphosphate (GS-443902) in normal human bronchial epithelial (NHBE) cells following pulsed (open red circle) and continuous (filled red circle) incubation. Data are adapted from Mackman et al. J. Med. Chem. (2021) (37) and are represented as the mean of at least 2 independent replicates. The dotted line at 10.6 pmol/million cells corresponds to the
mean C24 of triphosphate formed by RDV in SARS-CoV-2-infected HAE cells when the EC50 was determined to be 10 nM, as reported in Pruijssers et al. Cell Rep. (2020) (5).

Figure 3. Humans exhibit higher hepatic extraction of McGuigan prodrugs compared to non-human primates. (A) Doses of RDV trialed in the SAD and MAD arms of the phase 1 trial for a 50-70 kg human compared to the dose administered in a 3.6-5.7 kg NHP. The human and NHP doses have been converted to mg/kg and mg, respectively. Values were obtained from Humeniuk et al. Clin. Transl. Sci (2020) (31), Warren et al. Nature (2016) (21), and Williamson et al. Nature (2020) (11). (B) ALT and AST levels in NHP (rhesus macaques; N=6 per group) challenged with EBOV and treated with RDV for a total of 12 days adapted from Warren et al. Nature (2016). Regions shaded in grey correspond to normal ALT/AST ranges in NHP (ALT: 5-61 U/L; AST: 12-63 U/L) (57). NHP treated at 10 mg/kg for 12 days (light blue) do not experience significant elevations in ALT/AST. *No ALT/AST values were obtained at 14 days post infection (dpi) in vehicle control animals because 100% of animals had died. (C) Human equivalent dose (HED) of NHP RDV doses tested in Warren et al. Nature (2016) (8) via direct conversion and allometric scaling as described in West and Brown J. Exp. Biol. (2005) (41). Allometric scaling (1) uses an exponent of 0.75 while allometric scaling (2) uses an exponent of 0.80. HEDs were calculated for rhesus macaques weighing 3 kg (light grey) and 5 kg (dark grey) using Clymer Allometric Scaling calculator. (D) Sofosbuvir (SOF) is another McGuigan prodrug that is more readily metabolized in PHH compared to primary monkey hepatocytes in vitro (left) and in vivo (right). Left: levels of SOF NTP in primary hepatocytes following 2 h pulse of 10 µM
SOF. Right: levels of SOF NTP in human explanted livers compared and in preclinical model species at the allometrically scaled human dose (400 mg); open bars = $C_{\text{max}}$, shaded bars = $C_{24}$. $C_{24}$ in primary monkey livers was below the detection threshold. Values were re-plotted from Babusis et al. *Antimicrob. Agents Chemother.* (2018) (44) and Wang et al. *Drug Metab. Disp.* (2020) (43). Formation of SOF NTP is higher in PHH compared to primary monkey hepatocytes *in vitro* and *in vivo*, suggesting more efficient metabolism of McGuigan prodrugs in human compared to monkey livers.

**Figure 4.** Human equivalent dose of RDV in rhesus macaque models of COVID-19 or EBOV does not yield robust reductions in viral titers. (A-B) SARS-CoV-2-infected rhesus macaques ($2.6 \times 10^6$ TCID<sub>50</sub> nCoV-WA1-2020) treated with the allometrically scaled dose of 10 mg/kg 1 dpi followed by 5 mg/kg 2-6 dpi yields a modest global reduction in SARS-CoV-2 viral titers. Data adapted from the Supplementary Information of Williamson et al. *Nature* 2020(11). (A) Heat map of organs profiled for presence of SARS-CoV-2 RNA in RDV-treated (N=6) and CT (N=6) animals. LN = lymph node; UL = upper lobe; ML = middle lobe; LL = lower lobe. (B) Viral loads in nose swabs and throat swabs between RDV-treated and CT animals. (C) No significant decrease in viral loads in the upper and lower respiratory tracts in patients treated with RDV (200 mg day 1, 100 mg days 2-10) compared to placebo. Data are adapted from Wang et al. *Lancet* (2020) (2) and are represented as the mean (N=107, RDV; N=52, placebo). (D) Rhesus macaques were infected with EBOV (1,000 PFU, EBOV H. sapiens-tc/COD/1995/Kikwit) and treated IV with either the 10 mg/kg RDV at 2 dpi for 12 days (light blue), 10 mg/kg loading at 2 dpi then 3 mg/kg for 11 days (dark purple), or
10 mg/kg loading at 3 dpi then 3 mg/kg for 11 days (light purple), or vehicle (black).

Animals (N=6 per group) were monitored for a total of 28 days. The most distinct reductions in viral titers occurred for NHP in the 10 mg/kg treatment group (light blue).

(E) Survival curves for NHP at 28 dpi for treatment groups indicated. Though viral titers dropped to the limit of detection (LOD) for the 10/3 mg/kg D2 group (dark purple, D) by 12, only 2/6 NHP in this group survived (E, dark purple), skewing viral titer data. At the same time, this reinforces the close relationship between stark reductions in viral titers and improved disease outcomes, as the remaining 2/6 animals in the 10/3 mg/kg D2 group survived at 28 dpi. Data are adapted from Warren et al. *Nature* (2016) (21).

References


Food and Drug Administration. 2020. VEKLURY® (remdesivir).


<table>
<thead>
<tr>
<th>Cells</th>
<th>Tissue of origin</th>
<th>CC50 (µM)</th>
<th>SI (CC50/EC50, SARS-CoV-2)</th>
</tr>
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<tbody>
<tr>
<td>A549</td>
<td>Human lung cancer</td>
<td>650</td>
<td>6.5</td>
</tr>
<tr>
<td>Calu3</td>
<td>Human lung cancer</td>
<td>350</td>
<td>3.5</td>
</tr>
<tr>
<td>HAE</td>
<td>Human laryngeal carcinoma</td>
<td>1200</td>
<td>12.0</td>
</tr>
<tr>
<td>VeroE6</td>
<td>Human kidney cancer</td>
<td>2000</td>
<td>20.0</td>
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<tr>
<td>HEp-2</td>
<td>Human laryngeal carcinoma</td>
<td>600</td>
<td>6.0</td>
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<td>HepG2</td>
<td>Human liver cancer</td>
<td>340</td>
<td>3.4</td>
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<tr>
<td>PC-3</td>
<td>Human prostate cancer</td>
<td>890</td>
<td>8.9</td>
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<tr>
<td>PHH</td>
<td>Human hepatocytes</td>
<td>250</td>
<td>2.5</td>
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**Graph A**

- **X-axis**: NTP (pmol/million cells)
- **Y-axis**:log scale

**Graph B**

- **Legend**: A549, Calu3, HAE, VeroE6, HEp-2, HepG2, PC-3, PHH
- **X-axis**:log scale
- **Y-axis**:log scale
Figure 1. RDV is preferentially metabolized to active NTP in primary human hepatocytes. (A) NTP levels are roughly 2-fold higher in PHH compared to HAE cells. Cells were seeded at approximately 1x10^6 cells/well and were incubated with 1 µM RDV for 24 h. Mean of data replotted from Pruijsers et al. Cell Rep. (2020; HAE) and Xu et al. Antimicrob. Agents Chemother. (2020; PHH, HEP-2, PC-3). (B) Diverging EC50 values in various SARS-CoV-2-infected cells treated with RDV dramatically influences the selectivity index between infected and uninfected cells. EC_{50} values used (µM): A549=0.12; Calu3=0.28; HAE=0.010; VeroE6=1.65. EC_{50} values were obtained from Xie et al. Nat. Comm. (2020; A549) and Pruijsers et al. Cell Rep (2020; Calu3, VeroE6, HAE).
<table>
<thead>
<tr>
<th>Standard procedure</th>
<th>Incorrect Assumption</th>
<th>In Vivo Reality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Incubate cells with virus for 30-60 min.</td>
<td>1. Viral load is constant</td>
<td>1. Viral load is not always constant</td>
</tr>
<tr>
<td>2. Incubate cells with drug for 48-72+ hours</td>
<td>2. Target population of cells is constantly exposed to prodrug for 48-72+ hours at</td>
<td></td>
</tr>
<tr>
<td></td>
<td>supraphysiological [drug]</td>
<td>supraphysiological [drug]</td>
</tr>
<tr>
<td></td>
<td>2. Prodrug exposure can be short and is influenced by the T&lt;sub&gt;1/2&lt;/sub&gt; of the prodrug</td>
<td>2. Prodrug exposure can be short and is influenced by the T&lt;sub&gt;1/2&lt;/sub&gt; of the prodrug</td>
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<tr>
<td></td>
<td>3. [Drug] : target cell population is high in vivo</td>
<td>3. [Drug] : target cell population is low in vivo</td>
</tr>
<tr>
<td></td>
<td>4. Preferential extraction by other organs or cell types is negligible</td>
<td>4. Drug distribution can be uneven due to preferential metabolism by certain tissue over others</td>
</tr>
</tbody>
</table>
Table 1. Assumptions in standard cell culture procedures do not reflect the \textit{in vivo} behavior for prodrugs like RDV.
Prodrug activating enzyme expression in Vero E6 cells

![Graph showing enzyme expression](image)

<table>
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<tr>
<th>Prophylactic</th>
<th>Entry</th>
<th>Post-entry</th>
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<tbody>
<tr>
<td>Drug added and incubated for 1 h</td>
<td>Drug added and incubated for 1 h</td>
<td>Virus added</td>
</tr>
<tr>
<td>Virus added</td>
<td>Virus added</td>
<td>Incubate for 2 h</td>
</tr>
<tr>
<td>Incubate for 2 h</td>
<td>Incubate for 2 h</td>
<td>Drug added</td>
</tr>
<tr>
<td>Virus-drug mixture removed. Drug re-added, incubated for 48 h</td>
<td>Virus-drug mixture removed. Incubate for 48 h</td>
<td>Incubate for 48 h</td>
</tr>
</tbody>
</table>

RDV in NHBE cells

![Graph showing RDV in NHBE cells](image)

- Continuous RDV (48 h, 1 µM)
- Pulsed RDV (2 h, 1 µM)

EC$_{50, RDV}$ = 10 nM in HAE cells
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<table>
<thead>
<tr>
<th>Cohort</th>
<th>N</th>
<th>Dose (mg)</th>
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<th>Duration (days)</th>
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<td>SAD 5</td>
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<td>MAD 2</td>
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<table>
<thead>
<tr>
<th>NHP*</th>
<th>Dose (mg IV QD)</th>
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<tr>
<td>6</td>
<td>36-57 mg</td>
<td>10 mg/kg, 12 days</td>
<td>No significant elevations in ALT/AST</td>
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SARS-CoV-2 challenge study in NHP; Williamson et al. Nature (2020)

<table>
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<tr>
<th>NHP*</th>
<th>Dose (mg IV QD)</th>
<th>Maintenance dose (mg/kg)</th>
<th>Duration (days)</th>
<th>ALT/AST elevations</th>
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<td>36-57 mg loading, 15, 28.5 mg QD maintenance</td>
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<td>ND</td>
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**Table C**

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<th>NHP dose</th>
<th>Human equivalent dose (mg)</th>
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<td>3 mg/kg (3 kg)</td>
<td>150-210 Direct conversion, 74-96 Allometric scaling (1), 85-111 Allometric scaling (2)</td>
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<td>3 mg/kg (5 kg)</td>
<td>50-65 Direct conversion, 56-74 Allometric scaling (1), 59-74 Allometric scaling (2)</td>
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<tr>
<td>10/3 mg/kg (3 kg)</td>
<td>500-700, then 150-210 Direct conversion, 247-318, then 74-95, then 284-372 Allometric scaling (1), 284-372 Allometric scaling (2)</td>
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<tr>
<td>10/3 mg/kg (5 kg)</td>
<td>168-217, then 50-65, then 169-247, then 56-74 Allometric scaling (1), 169-247 Allometric scaling (2)</td>
</tr>
<tr>
<td>10 mg/kg (3 kg)</td>
<td>500-700 Direct conversion, 247-318, then 284-372 Allometric scaling (1), 284-372 Allometric scaling (2)</td>
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<tr>
<td>10 mg/kg (5 kg)</td>
<td>168-217 Direct conversion, 169-247 Allometric scaling (1), 169-247 Allometric scaling (2)</td>
</tr>
</tbody>
</table>

*Clinical dose: 200 mg, then 100 mg

**Figure B**

Transaminase levels in EBOV-challenged NHP treated with RDV for 12 days

**Figure D**

SOF NTP in primary hepatocytes in vitro

Liver SOF NTP in vivo at human equivalent dose

*Rhesus macaque weighs about 3.6-5.7 kg
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