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AUTHOR REPLY

Reply to Yan and Muller, "Single-Cell RNA Sequencing Supports Preferential Bioactivation of Remdesivir in the Liver"

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In their commentary "Single-Cell RNA Sequencing Supports Preferential Bioactivation of Remdesivir in the Liver," Yan and Muller state that two key pieces of data were misrepresented in our recent article titled "Key Metabolic Enzymes in Remdesivir Activation in Human Lung Cells" (9). We thank Yan and Muller for careful review of our publication and would like to address their comments as follows.

First, Yan and Muller stated that we omitted the fact that liver CES1, CatA, and HINT1 enzymes were involved in remdesivir (RDV) metabolism. In the paper, our intent was to confirm the role and characterize the specificity of the enzymes expected to be responsible for RDV activation in lung. Our study was not focused on the assessment of tissue distribution and organ accumulation of RDV metabolites. While this is still an important point, it should be a subject of independent studies, as different experimental approaches would be required (i.e., *in vivo* studies) to address this question. In Fig. 5 of our manuscript, we directly compared the protein expression of the three enzymes in the lung and liver S9 fractions by Western blot analysis, which showed that CES1 expression is significantly higher in liver than lung and that the expression of CatA and HINT1 is comparable between the two organs. Based on a thorough biochemical assessment of enzymatic kinetics for the hydrolysis of RDV, our data indicate that the CatA enzyme plays a major role in RDV metabolism in the lung since it showed >1,350-fold higher catalytic efficiency in hydrolyzing RDV than the CES1 enzyme. It has been clearly established that RDV is effectively activated and exerts potent antiviral activity against SARS-CoV-2 in primary human lung epithelial cells (1, 2). RDV is administered by intravenous infusion over 30 to 60 min, and it first flows into the heart followed by the target organ of the lungs before reaching the liver for metabolic extraction. Therefore, lungs are exposed to sufficient levels of RDV that can then be metabolized to its active triphosphate, independent of extraction in the liver.

Second, Yan and Muller claimed that we ignored the individual lung cell types and differential cell type-dependent expression of the metabolic enzymes and treated the human lung as a single homogenous tissue. They further concluded that the high expression of CES1, CTSA, and HINT1 genes was largely restricted to alveolar macrophages but was low in AT2 cells that are known to be involved in SARS-CoV-2 infection and replication. This is a misinterpretation of our analysis of the single-cell RNA sequencing (scRNAseq) data because, in our report, we pointed out that CES1, CTSA, and HINT1 genes are highly expressed in macrophages, which is consistent with Yan and Muller's comment. It should also be noted that our analysis of scRNAseq data showed that the same enzymes are abundantly expressed in lung epithelial cells, including AT2. In our study, we analyzed transcriptomes from a total of 65,662 cells with an average of 1,978 genes and 8,369 unique molecular identifiers (UMIs) detected per cell from the human lung, which allowed for comprehensive investigation of the gene expression patterns in 58 cell populations in a cell type-specific manner in human lung (3). In contrast, the conclusion that "the high expression of CES1, CTSA, and HINT1

Citation Murakami E, Bilello J, Li R, Li L, Porter D, Humeniuk R, Mackman R, Cihlar T, Osinusi A, Feng J. 2021. Reply to Yan and Muller, "Single-cell RNA sequencing supports preferential bioactivation of remdesivir in the liver." Antimicrob Agents Chemother 65:e01394-21. https://doi.org/10.1128/AAC.01394-21.

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This is a response to a letter by Yan and Muller https://doi.org/10.1128/AAC.01333-21.

Accepted manuscript posted online 9 August 2021

Published 17 September 2021

is largely restricted to alveolar macrophages" made by Yan and Muller was based on a more limited data set from the Human Protein Atlas (HPA), where the human lung scRNAseq data only included 4,599 cells from a public data set, with an average of 548 genes and 940 UMIs detected per cell (https://www.proteinatlas.org/about/assays+annotation) (4). This data set has lower sensitivity and is more likely to result in false negatives for the purpose of the intended analysis. Importantly and as pointed out above, the efficiency of RDV activation cannot be judged solely by the enzyme expression levels, and the specific catalytic efficiency of each enzyme participating in the activation of RDV needs to be also taken into account. In addition, it is not presently clear how the intracellular activation of RDV is affected by levels of activating enzymes in different cell types and what absolute level of each enzyme is sufficient to generate effective intracellular triphosphate concentrations to inhibit SARS-CoV-2 replication.

Finally, RDV continues to be the only approved direct antiviral for hospitalized COVID-19 patients. Data from randomized clinical trials, including ACTT-1, demonstrated that RDV is associated with clinical benefits (5). Emerging data, including the recently presented real-world evidence from almost 100,000 hospitalized patients, the largest RDV data set analyzed so far, shows that RDV is associated with clinical benefits, including a significant reduction in mortality rate (6–8). As such, these clinical data sets support the underlying premise that there is effective metabolism of RDV in the target tissues relevant for SARS-CoV-2 replication.

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