Reviewer’s report

Title: Prevalence of naturally occurring NS5A resistance-associated substitutions in patients infected with hepatitis C virus subtype 1a, 1b, and 3a, co-infected or not with HIV in Brazil

Version: 0 Date: 12 Jun 2017

Reviewer: Fiona McPhee

Reviewer's report:

Fernanda de Mello Malta et al. describe the prevalence of NS5A RAVs in 257 Brazilian patients who were either HCV mono-infected or HIV/HCV co-infected with either HCV GT-1a, GT-1b or GT-3a and were enrolled at a single hospital in Sao Paulo. Although sample size is limited (n=46), it is the only study describing NS5A polymorphisms in HCV GT-3a-infected patients from Brazil, however, small datasets have been described for GT-1 NS5A RAVs in patients from Brazil (2015). Since many patients infected with HCV GT-1a, -1b or -3 have now been treated with different DAA-based regimens in Brazil, it would be more informative to know the impact of the observed baseline NS5A polymorphisms on treatment outcome rather than speculating on their impact by referencing, in a number of cases, in vitro or in vivo monotherapy data.

General comments: Although "RAV" has been used historically, there has been an effort over the last year to use "resistance-associated substitution" or "RAS." The authors may want to consider replacing RAV with RAS. The authors use subtype 1a, subtype 1b, and subtype 3a, or HCV-1a or genotype 1a, etc. They may want to consider being consistent and using something like GT-1a, GT-1b, and GT-3a.

Major comments: Phylogenetic analyses of HCV NS5A sequences should be considered to determine sub-grouping of GT-1a, GT-1b, and GT-3a NS5A sequences, respectively, versus those described in other reports/public database to establish any difference in clustering of the sequences from patients in and around Sao Paulo. More attention should to be given to the baseline NS5A polymorphisms that have demonstrated impact on virologic outcome in treated patients. That kind of information would be more relevant for today's combination regimens as opposed to referencing data from proof-of-concept in vitro and in vivo monotherapy studies. Some of the cited studies are comparing the prevalence of NS5A RAVs in small cohorts of patients. There are limitations with these analyses that should be noted, depending on the patient population assessed. There are much larger studies that have been published in 2015 and 2016 that look at the prevalence of these NS5A RAVs in Europe, Asia, and the US. In the discussion, the NS5A polymorphisms are described with respect to fold resistance in vitro; this needs to be put into context given the pM activity of NS5A inhibitors. It should be noted that certain baseline NS5A polymorphisms conferring low level resistance may lower the resistance barrier while higher level resistance NS5A polymorphisms may have no impact on response rates depending on the patient population and regimen. Table 1 should be updated noting clinically relevant emergent NS5A substitutions especially since the described NS5A inhibitors are approved drugs (1 to 3 years). The relevance of 2-fold resistance in vitro for a pM NS5A inhibitor is unclear. Certain reports have used anywhere from >100-fold to >1000-fold resistance. What may be more pertinent is the impact that some of these baseline polymorphisms may have on the resistance pathway to failure of the NS5A inhibitors when combined with other DAAAs.

Minor comments: p4: Study population: Did any of these enrolled patients subsequently receive treatment? p5 Line 88: Was the genotype
first determined using a commercial kit? p5 Line 106/7: rephrase from a grammatical prospective. P5 Sanger sequencing: Was a sequencing threshold used to determine the reported NS5A polymorphisms by population-based sequencing? If so, please provide the cutoff. Use of different thresholds (or manual determination by eye) can lead to a variability in reported prevalence values, especially when the number of sequences are few (eg: there are only 9 HIV/HCV GT-1b and 15 GT-3a patients) so one or two patients can change the reported prevalence percentages. p5 Line 123-126: The authors should be aware of a summary of the GT-1a and GT-1b NS5A RAVs observed in the clinical setting to NS5A inhibitors, as well as available phenotypic data for approved NS5A drugs are described in Lontok et al (Hepatology. 2015 Nov;62(5):1623-32). Although the authors’ current references indicate the signature NS5A amino acid positions associated with virologic failure to an NS5A inhibitor, Table 1 shows emergent substitutions that have only been observed in vitro for some of the drugs. Either the text should be modified for clarity or Table 1 and its references should be updated; for example, deletions at P29 or P32 have been observed in GT-1b virologic failures rather than P32L/S for some NS5A inhibitors. Certain substitutions at positions 24, 62 and 92 have also been associated with failure to some NS5A inhibitors. For GT-1b and GT-3a, L31I has been reported to emerge but is not included in Table 1 (GT-1b: Manns M et al, Lancet 2014; GT-3a: Nelson DR et al, Hepatology 2015). The authors should check out the different drug labels to see what has been reported. p5 Line 126-127: The statement regarding the importance of Y93H is too simplistic. The impact of Y93 substitutions is dependent on genotype (and subtype), and DAA regimen. For example, moderate to high level resistance has not been reported for DCV (12-fold) or OMV (77-fold) in GT-1b although a greater level of resistance has been reported elsewhere for LDV. These levels of DCV and OMV resistance are low since their in vitro EC50 values are still sub-nM given the low pM potency of these inhibitors against wild-type strains. However, the combination of NS5A RAVs at L31 and Y93H confers high level resistance for these NS5A drugs. Furthermore, in GT-1a, Y93N confers high level resistance, however, other RAVs (for example, Q30E/H/K/R rather than Q30L) have been reported to be more relevant in DCV-based treatment failures. p7 Line 144: Need to make it clear whether the described percentages are looking at NS5A polymorphisms at 28, 30, 31 or 93 or the extended list of NS5A polymorphisms (32, 58, [24, 62, 93]). Assume it's the extended list of 32 and 58, which is inconsistent with literature and drug labels. p8 Line 188: Since there are only 257 patients, please only quote percentages to one decimal place. p8 Line 203: The authors should make it clear that the impact of baseline NS5A polymorphism on virologic outcome may be regimen- and/or duration-specific. Moreover, the combination of NS5A polymorphisms and cirrhosis status can also play a role. p9-10 Lines 223-234. The authors state their observations are contrary to some of the other reported studies. This could indeed be related to geographical differences in NS5A sequences, but it could also be compounded by small sample sizes described in some of the referenced studies and this study. p10 from Line 238: The role of M28V is not totally clear. For most NS5A inhibitors M28V does not confer resistance. Even for OMV, the level of resistance is low considering wild-type activity is in the single digit pM range in vitro. Thus, this sentence should be modified since M28V does not confer high level resistance to OMV. NS5A-P32L has not been reported to emerge in any of the DCV-based clinical studies, whether patients were infected with GT-1a or GT-1b. The prevalence of this polymorphism at baseline appears to be higher in the 41 mono-infected Brazilian patients compared to what has been observed in large clinical studies; this discrepancy could be a consequence of a limited sample size or related to region although it was not observed in the HIV/HCV co-infected patients. What is known about the two patients with P32L at baseline? Are the sequences similar by phylogenetic
analysis? GT-1a substitutions at NS5A-Y93 are also not created equally. The substitution conferring very high level resistance is Y93N while the impact of Y93H may depend on the regimen. Y93H against OMV confers much higher resistance than against LDV or DCV. Cirrhosis status appears to also play a role for some regimens. p10 Line 241: The combination of L31 +Y93H in GT-1b patients confers high level resistance. Each of these substitutions alone do not confer high level resistance. In most cases, it's minimal to low.p10 Line 244: Y93H is the signature substitution and this confers high level resistance in patients infected with HCV GT-3a. There are published articles that have shown that A30K alone does not appear to impact response rates depending on the DCV-based regimen and GT-3a patient population.p10 Line 248: This statement is not true. The authors should review the data. Q30 substitutions do confer resistance to varying degrees depending on the NS5A inhibitor. There are some substitutions, such as Q30L that confer minimal resistance.p10 Line 249-250: The level of resistance needs to be put in context when these inhibitors have EC50 values in the single digit pM range against wild-type sequences. Thus, 100-fold resistance can be in the pM range.p10 Line 253-256: Sentence requires rewording to more clearly state that although GT-1b NS5A-P58S substitutions by themselves confer no to minimal resistance to NS5A inhibitors, combination with NS5A RAVs such as L31 + Y93H substitutions at failure can significantly enhance drug resistance.p11 Lines 258-263: The sample sizes in this manuscript are too small to determine any meaningful difference in the prevalence of baseline NS5A polymorphisms by infection and genotype (subtype).p11 Line 272: It should state "Drugs with low resistance barriers" rather than "genetic resistance barriers". Even SOF has a low genetic resistance barrier (Are the methods appropriate and well described?)

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

No

Are the conclusions drawn adequately supported by the data shown?
If not, please explain in your comments to the authors.

No

Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?
If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

I am able to assess the statistics

Quality of written English
Please indicate the quality of language in the manuscript:

Acceptable
Declaration of competing interests

Please complete a declaration of competing interests, considering the following questions:

1. Have you in the past five years received reimbursements, fees, funding, or salary from an organisation that may in any way gain or lose financially from the publication of this manuscript, either now or in the future?

2. Do you hold any stocks or shares in an organisation that may in any way gain or lose financially from the publication of this manuscript, either now or in the future?

3. Do you hold or are you currently applying for any patents relating to the content of the manuscript?

4. Have you received reimbursements, fees, funding, or salary from an organization that holds or has applied for patents relating to the content of the manuscript?

5. Do you have any other financial competing interests?

6. Do you have any non-financial competing interests in relation to this paper?

If you can answer no to all of the above, write 'I declare that I have no competing interests' below. If your reply is yes to any, please give details below.

I am currently an employee of Bristol-Myers Squibb. Although the Discovery Virology Department no longer exists, the company has an HCV NS5A inhibitor (daclatasvir) that is a marketed product.

I agree to the open peer review policy of the journal. I understand that my name will be included on my report to the authors and, if the manuscript is accepted for publication, my named report including any attachments I upload will be posted on the website along with the authors' responses. I agree for my report to be made available under an Open Access Creative Commons CC-BY license (http://creativecommons.org/licenses/by/4.0/). I understand that any comments which I do not wish to be included in my named report can be included as confidential comments to the editors, which will not be published.

I agree to the open peer review policy of the journal.