

**Article title**

Absorption, distribution, metabolism, and excretion of the novel helicase-primase inhibitor, amenamevir (ASP2151), in rodents

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**Caption**

Electronic supplementary material 2

Chemical structure estimation of amenamevir metabolites

An authentic standard of amenamevir in CD<sub>3</sub>OD and CD<sub>3</sub>CN was analyzed by NMR and the chemical shifts are summarized in Supplementary Tables 1 and 2. Chemical structures of the isolated R1 to R10 were elucidated using LC/MS and NMR data as described below.

**R1**

The full scan MS of R1 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  515, which was 32 u larger than that of amenamevir (molecular mass of amenamevir: 482.55 Da). Hydroxylation at two positions of amenamevir might explain this observation. Comparison of <sup>1</sup>H NMR data of R1 with those of amenamevir in CD<sub>3</sub>CN showed that the proton signal of the methyl groups at  $\delta_H$  2.31 had disappeared and proton signals at  $\delta_H$  4.54 and 4.66 had appeared. In addition, the proton signals at  $\delta_H$  4.54 and 4.66 correlated with the carbon signal at  $\delta_C$  61 in the HSQCAD spectrum of R1. The carbon chemical shifts suggested the presence of methylene groups adjacent to the oxygen atoms in R1. To elucidate the position of these methylene groups, a NOESY experiment was conducted. Signals of these methylene groups correlated with those of the 1,2,3-trisubstituted benzene (i.e. benzene ring of the dimethylbenzene moiety) in the NOESY spectrum of R1. Based on these results, R1 was estimated to be the amenamevir metabolite where both of the two methyl groups in the dimethylbenzene moiety of amenamevir are hydroxylated.

## R2

In the LC/MS analysis immediately after purification, a single prominent peak was observed on the UV-chromatogram, and a molecular-related ion was detected at  $m/z$  535. However, during the LC/MS analysis after sample storage, several peaks were observed on the UV-chromatogram, molecular-related ions were detected at RT of 16.58 min ( $m/z$  495 and  $m/z$  513) and 17.98 min ( $m/z$  513). Therefore, it was suspected that R2 had changed between the first analysis and the second analysis. Similar to the observation in the LC/MS analysis, the  $^1\text{H}$  NMR spectrum in  $\text{CD}_3\text{OD}$  also changed across several days. We continued to elucidate the major component of the sample using the major signals that were consistently observed in separate NMR analyses. When the  $^1\text{H}$  NMR data were compared with those of amenamevir, chemical shifts of protons from the oxadiazole and 1,4-disubstituted benzene were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to the major component. Other aromatic protons were observed at  $\delta_{\text{H}}$  7.54,  $\delta_{\text{H}}$  7.64, and  $\delta_{\text{H}}$  7.66. Decoupling experiments showed that these protons were in the same spin system, which indicated that they were 1,2,3-trisubstituted benzene (i.e. benzene ring of the dimethylbenzene moiety) protons. Considering that the chemical shift values of the three proton signals were different from each other, and that the signals derived from the two methyl groups in the dimethylbenzene of amenamevir were not observed in the HSQC spectrum of the sample, it was presumed that the two methyl groups of amenamevir were metabolized differently. The HSQC experiment indicated the presence of a methylene group ( $\delta_{\text{H}}$  4.73,  $\delta_{\text{C}}$  60) and a methine group ( $\delta_{\text{H}}$  5.77,  $\delta_{\text{C}}$  101). In addition, an HMBC experiment revealed long-range  $^1\text{H}$ - $^{13}\text{C}$  correlations (a) between the methylene proton at  $\delta_{\text{H}}$  4.73 and the 1,2,3-trisubstituted benzene carbons at  $\delta_{\text{C}}$  131,  $\delta_{\text{C}}$  139, and  $\delta_{\text{C}}$  140 and (b) between the methine proton at  $\delta_{\text{H}}$  5.77 and the 1,2,3-trisubstituted benzene carbons at  $\delta_{\text{C}}$  128,  $\delta_{\text{C}}$  136, and  $\delta_{\text{C}}$  139. Taking the chemical shift values of the methylene and methine protons into consideration, the major component was estimated to contain a hydroxymethyl group ( $\delta_{\text{H}}$  4.73,  $\delta_{\text{C}}$  60) and a dihydroxymethyl group ( $\delta_{\text{H}}$  5.77,  $\delta_{\text{C}}$  101) in the 1,2,3-trisubstituted benzene. This chemical structure suggested that the molecular mass of the major component was 530 Da, which is consistent with the aforementioned LC/MS data. Specifically, the molecular-related ions of  $m/z$  513 and  $m/z$  495 at RT of 16.58 min observed after storage could be  $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$  and  $[\text{M}+\text{H}-2\text{H}_2\text{O}]^+$ , respectively, of the major component. The molecular-related ion of  $m/z$  513 at RT of 17.98 min observed after storage could be  $[\text{M}+\text{H}]^+$  of R2, if R2 has a formyl group and is hydrated to produce the major component. The molecular-related ion at  $m/z$  535 observed immediately after purification could be  $[\text{M}+\text{Na}]^+$  of R2. Therefore, R2 was estimated to be the amenamevir metabolite where one methyl group in the dimethylbenzene was hydroxylated, and the other methyl group in the dimethylbenzene was oxidized to a formyl group.

### R3

The full scan MS of R3 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  675, which was 192 u larger than that of amenamevir. The ion at  $m/z$  675 produced a 176 u smaller ion in the product ion scan, which indicated glucuronidation (+176 u) in the metabolism of amenamevir to R3. The 16 u difference between 176 u and 192 u could be attributed to hydroxylation in the metabolism of amenamevir to R3. In the  $^1H$  NMR spectrum of R3, proton signals from the oxadiazole and 1,4-disubstituted benzene were observed at  $\delta_H$  9.23,  $\delta_H$  7.73, and  $\delta_H$  8.02. These chemical shifts were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R3. An HMBC experiment revealed the following correlations: (a) the methyl protons at  $\delta_H$  2.38 correlated with the 1,2,3-trisubstituted benzene carbons at  $\delta_C$  130–140. (b) the methylene group protons at  $\delta_H$  4.73/5.12 and  $\delta_H$  4.84/5.07 correlated with the 1,2,3-trisubstituted benzene carbons at  $\delta_C$  130–140. These HMBC correlations suggested that one methyl group of amenamevir retained while the other is oxidized to a hydroxymethyl group. A TOCSY experiment revealed a spin system containing  $\delta_H$  4.54,  $\delta_H$  3.79,  $\delta_H$  3.84,  $\delta_H$  3.20,  $\delta_H$  3.35,  $\delta_H$  3.45, and  $\delta_H$  3.50, suggesting that these signals originated from a glucuronic acid moiety. A NOESY spectrum of R3 showed that the anomeric protons of the glucuronic acid moiety at  $\delta_H$  4.54 correlated with the hydroxymethyl protons at  $\delta_H$  5.12. These HMBC, TOCSY, and NOESY correlations suggested that a methyl group in the dimethylbenzene moiety of amenamevir was hydroxylated and then conjugated with a glucuronic acid in the metabolism of amenamevir to R3. The coupling constant of the anomeric protons at  $\delta_H$  4.54 was 8 Hz, indicating that the glucuronic acid moiety was a  $\beta$ -anomer. Based on these results, R3 was estimated to be a O- $\beta$ -glucuronide resulting from hydroxylation at a methyl group in the dimethylbenzene moiety of amenamevir.

### R4

The full scan MS of R4 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  515, which was 32 u larger than that of amenamevir. Hydroxylation at two positions of amenamevir might explain this observation. Comparison of  $^1H$  NMR data of R4 with those of amenamevir showed that chemical shifts of protons from the oxadiazole and 1,4-disubstituted benzene were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R4. A proton signal around  $\delta_H$  2.3–2.4 from the methyl group(s) changed from s,6H to s,3H, suggesting metabolism at one of the two methyl groups. New signals were proton signals at  $\delta_H$  4.77 and  $\delta_H$  4.83, suggesting the presence of the methylene group adjacent to the oxygen atom. In the HMBC spectrum, the proton signals at  $\delta_H$  4.77 and  $\delta_H$  4.83 correlated with carbon at  $\delta_C$  139. These chemical shifts suggest that one methyl group of the dimethylbenzene moiety of amenamevir

was hydroxylated. Furthermore, a TOCSY experiment revealed a spin system containing  $\delta_{\text{H}}$  2.39,  $\delta_{\text{H}}$  4.29,  $\delta_{\text{H}}$  3.16,  $\delta_{\text{H}}$  2.91,  $\delta_{\text{H}}$  2.94,  $\delta_{\text{H}}$  2.89,  $\delta_{\text{H}}$  2.26, and  $\delta_{\text{H}}$  2.07, suggesting that these signals originated from three methylene groups and two methine groups at the thiane sulfone ring. It was thought that a methylene group in the thiane sulfone ring may have changed into a methine group. An HSQCAD experiment revealed a carbon and a proton in the new methine group at  $\delta_{\text{C}}$  68 and  $\delta_{\text{H}}$  4.29, respectively. These chemical shifts, along with the HMBC, TOCSY, and HSQCAD correlations, indicated hydroxylation of the thiane sulfone ring, including the hydroxylation position. Based on these results, R4 was estimated to be the amenamevir metabolite where one methyl group in the dimethylbenzene moiety of amenamevir, and the thiane sulfone ring of amenamevir were hydroxylated.

#### R5

The full scan MS of R5 produced a protonated molecule ( $\text{M} + \text{H}^+$ ) at  $m/z$  499, which was 16 u larger than that of amenamevir. Hydroxylation at one position of amenamevir might explain this observation. Comparison of  $^1\text{H}$  NMR data of R5 with those of amenamevir showed that chemical shifts of protons from the oxadiazole, 1,4-disubstituted benzene, and thiane sulfone ring were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R5. A proton signal around  $\delta_{\text{H}}$  2.3–2.4 from the methyl group(s) changed from s,6H to s,3H, suggesting metabolism at one of the two methyl groups. New signals were proton signals at  $\delta_{\text{H}}$  4.56 and  $\delta_{\text{H}}$  4.70. An HMBC experiment revealed long-range  $^1\text{H}$ - $^{13}\text{C}$  correlations between the methylene group protons at  $\delta_{\text{H}}$  4.56 and  $\delta_{\text{H}}$  4.70 and the benzene carbons at  $\delta_{\text{C}}$  128 and  $\delta_{\text{C}}$  140. Based on these results, R5 was estimated to be the amenamevir metabolite where a methyl group in the dimethylbenzene moiety of amenamevir was hydroxylated.

#### R6

The full scan MS of R6 produced a protonated molecule ( $\text{M} + \text{H}^+$ ) at  $m/z$  499, which was 16 u larger than that of amenamevir. Hydroxylation at one position of amenamevir might explain this observation. Comparison of  $^1\text{H}$  NMR data of R6 with those of amenamevir showed that chemical shifts of protons from the oxadiazole, 1,4-disubstituted benzene, and thiane sulfone ring were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R6. New doublet signals were observed at  $\delta_{\text{H}}$  6.78 and  $\delta_{\text{H}}$  7.02 in the aromatic region. These were attributed to the 1,2,3-trisubstituted benzene (i.e. benzene ring of the dimethylbenzene moiety). To explain the NMR signals, the dimethylbenzene moiety of R6 has to be asymmetric. Based on these results, R6 was estimated to be the amenamevir metabolite where the 2,6-dimethylphenyl group of amenamevir was changed to be a 3-hydroxy-2,6-dimethylphenyl group.

### R7

The full scan MS of R7 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  513, which was 30 u larger than that of amenamevir. Hydroxylation at two positions and removal of two hydrogens of amenamevir might explain this observation. Comparison of  $^1H$  NMR data of R7 with those of amenamevir showed that chemical shifts of protons from the oxadiazole and 1,4-disubstituted benzene were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R7. A new singlet proton signal was observed at  $\delta_H$  10.55. This chemical shift suggested the presence of an aldehyde group in R7. As an HSQCAD experiment revealed a methyl signal consisting of  $\delta_H$  2.39,  $\delta_H$  2.50, and  $\delta_C$  18, it was presumed that at least one methyl group in the dimethylbenzene moiety was not changed. The TOCSY spectrum of R7 showed correlations between  $\delta_H$  2.0–4.3 (this observation is similar to R4), suggesting hydroxylation of the thiane sulfone ring. Based on these results, R7 was estimated to be the amenamevir metabolite where one methyl group in the dimethylbenzene moiety of amenamevir changed to aldehyde, and the thiane sulfone ring of amenamevir hydroxylated.

### R8

The full scan MS of R8 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  497, which was 14 u larger than that of amenamevir. Hydroxylation at one position and removal of two hydrogens of amenamevir might explain this observation. Comparison of  $^1H$  NMR data of R8 with those of amenamevir showed that chemical shifts of protons from the oxadiazole and 1,4-disubstituted benzene were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R8. A new singlet proton signal was observed at  $\delta_H$  10.35. This chemical shift suggests the presence of an aldehyde group. As an HSQCAD experiment revealed a methyl signal consisting of  $\delta_H$  2.35 and  $\delta_C$  18, it was presumed that at least one methyl group was not changed. Based on these results, R8 was estimated to be the amenamevir metabolite where one methyl group in the dimethylbenzene moiety of amenamevir changed to aldehyde.

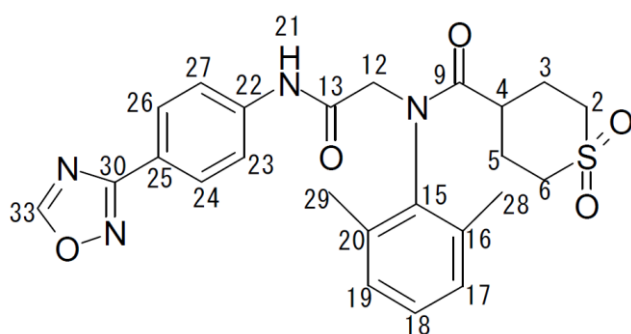
### R9

The full scan MS of R9 produced a deprotonated molecule ( $M - H^-$ ) at  $m/z$  511, which was 30 u larger than that of amenamevir. Hydroxylation at two positions and removal of two hydrogens of amenamevir might explain this observation. Comparison of  $^1H$  NMR data of R9 with those of amenamevir showed that chemical shifts of protons from the oxadiazole and 1,4-disubstituted benzene were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R9. In addition, a TOCSY experiment revealed that

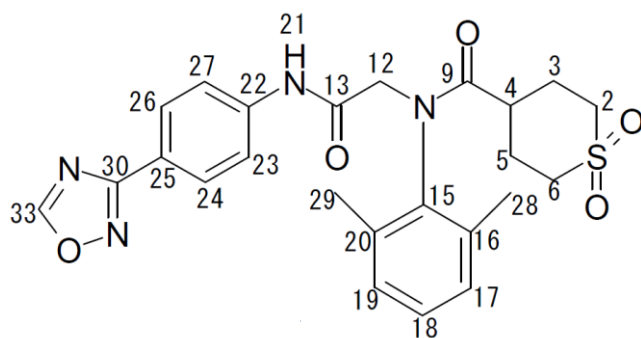
the thiane sulfone ring was intact. Unlike amenamevir, the TOCSY spectrum suggested an asymmetric 1,2,3-trisubstituted benzene at  $\delta_{\text{H}}$  7.39,  $\delta_{\text{H}}$  7.35, and  $\delta_{\text{H}}$  7.70. Furthermore, the NOESY spectrum showed a correlation between the protons of the 1,2,3-trisubstituted benzene at  $\delta_{\text{H}}$  7.39 (1H,d) and the methyl protons at  $\delta_{\text{H}}$  2.34. Based on these results, R9 was estimated to be the amenamevir metabolite where one methyl group in the dimethylbenzene moiety of amenamevir changed to a carboxylic acid.

#### R10

The full scan MS of R10 produced a protonated molecule ( $M + H^+$ ) at  $m/z$  457, which was 26 u smaller than that of amenamevir. Incorporation of two hydrogens to amenamevir and removal of one oxygen and one carbon of amenamevir might explain this observation. Comparison of  $^1\text{H}$  NMR data of R10 with those of amenamevir in  $\text{CD}_3\text{OD}$  showed that chemical shifts of protons from the 1,2,3-trisubstituted benzene, methyl groups, and thiane sulfone ring were similar to those of amenamevir, suggesting that the protons of these moieties were intact during the metabolism of amenamevir to R10. The oxadiazole proton at  $\delta_{\text{H}}$  9.23 in amenamevir was not observed in R10. In addition, proton signals in the 1,4-disubstituted benzene were shifted. Based on these results, R10 was estimated to be the amenamevir metabolite where the oxadiazole ring changed to an amidino group.

**Supplementary Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments of amenamevir in  $\text{CD}_3\text{OD}$ .

Position	$\delta_{\text{C}}$ (ppm)	$\delta_{\text{H}}$ (ppm)
2, 6	48	2.95, 3.14
3, 5	27	2.09, 2.28
4	38	2.47
12	54	4.28
13	167	
15	140	
16, 20	137	
17, 19	130	7.21
18	129	7.25
22	142	
23, 27	120	7.74
24, 26	128	8.04
25	122	
28, 29	15	2.39
30	167	
33	166	9.23

**Supplementary Table 2.**  $^1\text{H}$  NMR assignments of amenamevir in  $\text{CD}_3\text{CN}$ .

Position	$\delta_{\text{H}}$ (ppm)
2, 6	2.80, 3.01
3, 5	1.99, 2.02
4	2.40
12	4.19
17, 19	7.19
18	7.25
21	8.93
23, 27	7.72
24, 26	8.04
28, 29	2.31
33	8.98