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Metabolism and Pharmacokinetics of Novel Selective Vascular Endothelial Growth Factor Receptor-2 Inhibitor Apatinib in Humans

Juefang Ding, Xiaoyan Chen, Zhiwei Gao, Xiaojian Dai, Liang Li, Cen Xie, Haoyuan Jiang, Lijia Zhang, and Dafang Zhong

Drug Metabolism and Disposition

Supplemental Results

The Structural Elucidation of Apatinb Metabolites in Human Plasma, Urine, and Feces

A description of the information used for the assignment of each metabolite is detailed below.

Parent drug M0. A chromatographic peak at 11.4 min was detected in human plasma and feces, which showed a protonated molecular ion at *m/z* 398.197 and an elemental composition of $C_{24}H_{23}N_5O$. The retention time and MS fragmentation patterns were identical to those of the parent drug, indicating that this component was the unchanged parent drug, designated as M0.

M1. Accurate mass measurement of M1 showed a protonated molecular ion of 414.193, 16 amu higher than that of apatinib. The derived elemental composition of M1-1 was $C_{24}H_{23}N_5O_2$, suggesting that the molecule had undergone monooxygenation. The extracted ion chromatogram of *m/z* 414.193 showed six chromatographic peaks at 8.1 min (M1-1), 7.7 min (M1-2), 8.5 min (M1-3), 8.6 min (M1-4), 10.1min (M1-5), and 10.9 min (M1-6).

The high CE mass spectra of M1-1 and M1-2 showed fragment ions at *m/z* 387.190, 212.085, 184.091, and 92.052. The ion at *m/z* 387.190 was 16 amu higher than the fragment ion at *m/z* 371.187 of M0. Moreover, the ions at *m/z* 212.085, 184.091, and 92.052 were identical to those of M0, suggesting that parts B and C were intact. The structures of M1-1 and M1-2 were further confirmed by comparing the chromatographic behaviors and mass spectrometric characteristics with those of the synthetic authentic standards. The ^1H nuclear magnetic resonance (NMR) data of

M1-1 and M1-2 are listed in Table 2. Based on these data, M1-1 was unequivocally identified as *cis*-3-hydroxy-apatinib and M1-2 was confirmed as *trans*-3-hydroxy-apatinib.

The high CE mass spectra of M1-3 and M1-4 both showed a major fragment ion at *m/z* 212.085, which was identical to that of M0, indicating that the oxidation had occurred at part A. The definitive structures of M1-3 and M1-4 could not be determined based on the MS data.

The high CE mass spectrum of M1-5 showed major fragment ions at *m/z* 228.079 and 92.050. The fragment ion at *m/z* 228.079 was 16 amu higher than the major fragment ion at *m/z* 212.085 of M0, indicating that oxidation had occurred at part B. The exact structure of M5 needs to be further characterized.

The high CE mass spectrum of M1-6 showed fragment ions at *m/z* 397.191, 228.078, and 108.044. The ion at *m/z* 397.191 showed a loss of 17 amu (a hydroxyl radical) from the protonated molecular ion, suggesting that the molecule had undergone *N*-oxidation. The ions at *m/z* 228.078 and 108.044 were 16 amu higher than the fragment ions at *m/z* 212.085 and 92.050 of M0, respectively, indicating that oxidation had occurred at part C. Treatment of human plasma with a selective reductant of *N*-oxides $TiCl_3$ resulted in the disappearance of M1-6 and an increase in the response of the unchanged drug in the chromatogram. The retention time and mass spectral data of M1-6 were identical to those of a chemically synthesized reference standard using *m*-chloroperoxybenzoic acid as the selective oxidant. The ^{1}H NMR data of M1-6 is listed in Table 2. Based on these data, M1-6 was unequivocally

identified as apatinib-25-*N*-oxide.

M2. Metabolite M2 was eluted at 10.0 min. Accurate mass measurement of M2 provided a protonated molecular ion of 307.157. The derived elemental composition of M2 was $C_{18}H_{18}N_4O$, indicating a loss of C_6H_5N (picolyl moiety) from M0. High CE mass spectrum of M1-6 showed major fragment ion at *m/z* 121.039, indicating a loss of C_6H_5N (picolyl moiety) compared with the fragment ion at *m/z* 212.085 of M0. Metabolite M2 was identified as the *N*-dealkylated metabolite.

M3. Accurate mass measurement of M3 provided a protonated molecular ion of 323.157, 16 amu higher than that of M2. The derived formula of M3 was $C_{18}H_{18}N_4O_2$, suggesting that the molecule had undergone *N*-dealkylation and monooxygenation. The extracted ion chromatogram of *m/z* 323.1508 showed four chromatographic peaks at 6.2 min (M3-1), 6.6 min (M3-2), 7.1 min (M3-3), and 9.6 min (M3-4).

The high CE mass spectra of M3-1, M3-2, and M3-3 showed the same fragment ion at *m/z* 121.040, which was identical to that of M2, indicating that oxidation had occurred at part A. M3-1 was detected as a metabolite when synthesized M1-2 was incubated with HLMs in the presence of NADPH. Therefore, M3-1 was identified as the *N*-dealkylated metabolite of M1-2. M3-2 was likewise identified as the *N*-dealkylated metabolite of M1-1. The definitive structures of M3-3 could not be determined on the basis of MS data.

The high CE mass spectrum of M3-4 showed fragment ion at *m/z* 137.035, 16 amu higher than that of M2, indicating that oxidation had occurred at part B. The definitive structure of M3-3 could not be determined on the basis of MS data.

M4. Accurate mass measurement of M4 provided a protonated molecular ion of 339.144, 32 amu higher than that of M2. The derived formula of M4 was $C_{18}H_{18}N_4O_3$, suggesting that the molecule had undergone *N*-dealkylation and dioxygenation. The extracted ion chromatogram of *m/z* 339.144 showed nine chromatographic peaks at 4.5 min (M4-1), 5.1 min (M4-2), 5.6 min (M4-3), 5.8 min (M4-4), 6.2min (M4-5), 6.6 min (M4-6), 6.8 min (M4-7), 7.2 min (M4-8), and 7.7 min (M4-9).

The high CE mass spectra of M4-1, M4-2, M4-3, and M4-4 showed the same fragment ion at *m/z* 121.038, which was identical to that of M2, indicating that two oxygen atoms had been introduced into part A. The definitive structures of M4-1, M4-2, M4-3, and M4-4 could not be determined on the basis of MS data.

The high CE mass spectra of M4-5, M4-6, M4-7, M4-8, and M4-9 showed the same fragment ion at *m/z* 137.036, 16 amu higher than that of M2, indicating that one oxygen atom had been added to part B and another had been added to part A. the definitive structures of these molecules could not be determined on the basis of MS data. To determine the structures of M4-7 and M4-8, they were isolated from human urine samples as described under *Materials and Methods* for NMR analysis. The ^{1}H NMR data of M4-7 and M4-8 are listed in Table 2. Based on these data, M4-7 was unequivocally identified as *trans*-3-hydroxy-16-hydroxy-*N*-dealkylated metabolite, and M4-8 was identified as *cis*-3-hydroxy-16-hydroxy-*N*-dealkylated metabolite.

M5. Accurate mass measurement of M5 provided a protonated molecular ion of 412.180, 14 amu higher than that of M0. The derived elemental composition of M5 was $C_{24}H_{21}N_5O_2$, indicating that the molecule had undergone monooxygenation and

dehydrogenation. The extracted ion chromatogram of *m/z* 412.180 showed four chromatographic peaks at 7.1 min (M5-1), 7.4 min (M5-2), 7.8 min (M5-3), and 8.6 min (M5-4). The definitive structures of these molecules could not be determined on the basis of MS data.

M6. Accurate mass measurement of M6 provided a protonated molecular ion of 430.195, 32 amu higher than that of M0. The derived elemental composition of M6 was $C_{24}H_{23}N_5O_3$, suggesting that the molecule had undergone dioxygenation. The extracted ion chromatogram of *m/z* 430.195 showed eleven chromatographic peaks at 5.7 min (M6-1), 6.2 min (M6-2), 6.5 min (M6-3), 6.7 min (M6-4), 6.8 min (M6-5), 7.1 min (M6-6), 7.2 min (M6-7), 7.6 min (M6-8), 7.8 min (M6-9), 8.1 min (M6-10), and 8.5 min (M6-11).

The high CE mass spectra of M6-1, M6-2, M6-3, M6-4, and M6-6 all showed the same major fragment ion at *m/z* 212.088, which was identical to that of M0, indicating that the two oxygen atoms had been both introduced into part A. Their definitive structures could not be determined on the basis of MS data.

The high CE mass spectra of M6-5, M6-7, and M6-8 all showed fragment ions at *m/z* 228.088 and *m/z* 92.053. The fragment ion at *m/z* 228.088 was 16 amu higher than the major fragment ion at *m/z* 212.085 of M0, and the fragment ion at *m/z* 92.053 was identical to that of M0, indicating that the two oxygen atoms had been introduced into parts A and B. Their definitive structures could not be determined on the basis of MS data.

The high CE mass spectra of M6-9, M6-10, and M6-11 all showed the same

fragment ion at *m/z* 108.0201, which was identical to that of M1-6, indicating that one of the two oxygen atoms had been introduced into part C. Treatment of human urine samples with $TiCl_3$ resulted in the disappearance of M6-9, M6-10 and M6-11 (data not shown). To determine the structures of M6-9 and M6-10, the molecules were isolated from human urine samples as described under *Materials and Methods* for NMR analysis. The ^1H NMR data of M6-9 and M6-10 are listed in Table 2. The data suggested that M6-9 and M6-10 were *trans*-3-hydroxy-25-*N*-oxide metabolite and *cis*-3-hydroxy-25-*N*-oxide metabolite, respectively. The definitive structure of M6-11 could not be determined on the basis of MS data.

M7. Accurate mass measurement of M7 provided a protonated molecular ion of 494.151, 96 amu higher than that of M0. The derived elemental composition of M7 was $C_{24}H_{23}N_5O_5S$, suggesting that the molecule had undergone monooxygenation and sulfate conjugation. The extracted ion chromatogram of *m/z* 414.151 showed two chromatographic peaks at 7.0 min (M7-1) and 7.3 min (M7-2). The high CE mass spectra of M7-1 and M7-2 both showed the same major fragment ion at *m/z* 212.081, which was identical to that of M0, indicating that oxidation and sulfate conjugation had occurred at part A. M7-1 and M7-2 were tentatively identified as sulfate conjugates of hydroxylated apatinib. Complete structure assignment of M7-1 and M7-2 was not possible based on MS data alone.

M8. Accurate mass measurement of M8 provided a protonated molecular ion of 499.174, 101 amu higher than that of M0. The derived elemental composition of M8 was $C_{24}H_{26}N_4O_8$, suggesting that the molecule had undergone monooxygenation,

glucuronide conjugation, and *N*-dealkylation (loss of the picolyl moiety). The extracted ion chromatogram of *m/z* 499.174 showed two chromatographic peaks at 5.1 min (M8-1) and 5.5 min (M8-2).

The high CE mass spectra of M8-1 and M8-2 both showed fragment ions at *m/z* 323.151 and 121.038. The fragment ion at *m/z* 323.151 was 176 amu lower than its protonated ion, corresponding to the loss of a glucuronide moiety. The fragment ion at *m/z* 121.038 was identical to that of M2, indicating that part A was the site of oxidation. Treatment of human urine samples with β-glucuronidase resulted in the disappearance of M8-1 and M8-2 in the chromatogram (data not shown). M8-1 was detected as a metabolite when synthesized M1-2 was incubated with HLMs in the presence of NADPH and UDPGA. Therefore, M8-1 was identified as *trans*-3-hydroxy-*O*-glucuronide-*N*-dealkylated metabolite. M8-2 was likewise identified as the *cis*-3-hydroxy-*O*-glucuronide-*N*-dealkylated metabolite. M8-2 was isolated from human urine samples as described under *Materials and Methods*. The ^{1}H NMR data of M8-2 is listed in Table 2.

M9. Accurate mass measurement of M9 provided a protonated molecular ion of 590.220, 192 amu higher than that of unchanged drug. The derived elemental composition of M9 was $C_{30}H_{31}N_5O_8$, suggesting that the molecule had undergone monooxygenation and glucuronide conjugation. The extracted ion chromatogram of *m/z* 590.220 showed two chromatographic peaks at 6.5 min (M9-1) and 6.8 min (M9-2).

The high CE mass spectra of M9-1 and M9-2 both showed fragment ions at *m/z*

414.195 and 212.082. The fragment ion at *m/z* 414.195 was 176 amu lower than its protonated ion, corresponding to the loss of a glucuronide moiety. The fragment ion at *m/z* 212.082 was identical to that of M1-1 to M1-4, indicating that part A was the site of oxidation. Treatment of human plasma samples with β-glucuronidase resulted in the disappearance of M9-1 and M9-2 in the chromatogram (data not shown). M9-1 was detected as a metabolite when synthesized M1-2 was incubated with HLMs in the presence of UDPGA. Therefore, M9-1 was identified as the *O*-glucuronide of *trans*-3-hydroxy-apatinib. M9-2 was likewise identified as the as the *O*-glucuronide of *cis*-3-hydroxy-apatinib. M9-2 was isolated from human urine samples as described under *Materials and Methods*. The ^{1}H NMR data of M9-2 is listed in Table 2.

M10. Metabolite M10 was eluted at 6.9 min. Accurate mass measurement of M10 provided a protonated molecular ion of 606.210, 208 amu higher than that of unchanged drug. The derived elemental composition of M10 was $C_{30}H_{31}N_5O_9$, suggesting that the molecule had undergone dioxygenation and glucuronide conjugation. The high CE mass spectrum of M10 showed fragment ions at *m/z* 430.1832, 176 amu lower than its protonated ion, corresponding to the loss of a glucuronide moiety. The definitive structure of M10 could not be determined on the basis of MS data.