**Title: Absorption, Distribution, Metabolism, and Excretion (ADME) of Capmatinib (INC280) in Healthy Male Volunteers and In Vitro Aldehyde Oxidase Phenotyping of the Major Metabolite**

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**Supplementary Material**

## 1. Detection of radioactivity in blood, plasma, urine, and feces

Liquid scintillation counting was used to measure radioactivity in blood, plasma, urine, and feces, with typical counting times of 10 or 30 min. Low levels of radioactivity in blood and plasma were counted for 60 or 120 min. Blood samples (triplicates of 300 μL each, weighed) were measured after solubilization with tissue solubilizer (SolvableTM [PerkinElmer, NL]), decolorization with hydrogen peroxide and mixing with liquid scintillation cocktail. Plasma samples (triplicates of 250 μL each, weighed) were mixed directly with the liquid scintillation cocktail before radiometry. Feces samples (quadruplicates of ~0.5 g each, weighed) were measured after combustion. Urine samples (duplicates of 1 mL each) were measured after mixing with the cocktail. The limit of quantification (LOQ) for blood (30 or 120 min counting time) and plasma (10, 60, or 120 min counting time) samples were 50 and 30 dpm/g corresponding to 91 and 54 ng-eq/g, respectively, while for urine and feces samples (10 min counting time) LOQ was 10 and 40 dpm/g corresponding to 18 and 73 ng-eq/g, respectively.

Plasma samples: In triplicate, an aliquot of 250 μL plasma sample (weight was determined at the clinical site) was transferred into 20 mL glass vials (PerkinElmer, NL), and then a 10 mL scintillation cocktail (Ultima GoldTM [PerkinElmer, NL]) was added. After vortex mixing for at least 5 seconds, the sample was placed in the liquid scintillation counter for at least 30 min prior to counting. The total 14C-radioactivity of the samples was determined by counting the samples until a statistical error (2s) of 0.5% was obtained with a maximum counting time of 10 min. Some samples were re-counted using a count time of maximal 120 min. Quantitation was achieved using an external 20 μCi (740 kBq) 133Ba radiation source (external standard method) and based on a quench curve.

Whole blood samples: In triplicate, an aliquot of 300 μL whole blood sample (weight was determined at the clinical site) was transferred into a 20 mL glass vial. 1.00 mL of the tissue solubilizer (SolvableTM [PerkinElmer, NL]) was added and the sample was incubated for 60 min at 60°C in a water bath. After cooling in a cold water bath, 100 μL of 0.1 M titriplex (Merck, NL) was added, and the sample was decolorized by adding three times a volume of 75.0 μL hydrogen peroxide (Merck, NL) in steps of 5 min. After incubation for 15 min at room temperature, the mixture was heated again for 15 min at 45°C in a water bath, followed by 30 min at 60°C. After cooling in a cold water bath, 18 mL of the scintillation cocktail (Ultima GoldTM (PerkinElmer, NL) was added. After vortex mixing for at least 5 seconds, the vial was placed in an ultra-sonication bath at room temperature for 5 min, with the cap of the vial loosely closed. The vial was placed in the liquid scintillation counter for at least 36 h prior to counting. The total 14C-radioactivity of the samples was determined by counting the samples until a statistical error (2s) of 0.5% was obtained with a counting time of 30 min. Some samples were re-counted using a count time of maximal 120 min. Quantitation was achieved using an external 20 μCi (740 kBq) 133Ba radiation source (external standard method) and based on a quench curve.

Urine samples: In duplicate, 1000 μL urine sample was transferred to a 7 mL glass vial (PerkinElmer, NL) and weighed, and 5 mL scintillation cocktail [Ultima GoldTM (PerlinElmer, NL)] was added. After vortex mixing for at least 5 seconds, the sample was placed in the liquid scintillation analyzer at least 30 min before counting. The total 14C-radioactivity of the samples was determined by counting the samples until a statistical error (2s) of 0.5% was obtained with a counting time of 10 min. The validated range is 10–50,000 dpm/mL for the Low Level Count Mode (LLCM) and 40,000–1,000,000 dpm/mL for the Normal Count Mode (NCM). Quantitation was achieved using an external 20 μCi (740 kBq) 133Ba radiation source (external standard method) and based on a quench curve.

Feces samples: Four accurately weighed aliquots of approximately 500 mg of the feces homogenate sample were dried in a stove at 50°C for at least 3 h. After the addition of 100 μL CombustaidTM (PerkinElmer, NL) to the dry homogenates, the samples were combusted in a sample oxidizer model 307 (PerkinElmer, NL). CarboSorb-ETM (PerkinElmer, NL) 7 mL was used as an absorber agent for carbon dioxide. At the end of the combustion cycle, the absorber was mixed with 13 mL of the scintillation cocktail PermaFluor ETM (PerkinElmer, NL). The samples were placed in the liquid scintillation analyzer for at least 60 min before counting.The total 14C-radioactivity of the samples was determined by counting the samples until a statistical error (2s) of 0.5% was obtained with a counting time of 10 min. The validated range is 40–160,000 dpm/g for the LLCM and 100,000–1,000,000 dpm/g for the NCM. Quantitation was achieved using an external 20 μCi (740 kBq) 133Ba radiation source (external standard method) and based on a quench curve.

**TABLE 1 Capmatinib and metabolites in plasma (mean of n=6, SD)**

Estimated AUC0-12h of capmatinib and metabolites in plasma following a single oral dose of 600 mg [14C]capmatinib; components listed in the order of elution

|  |  |  |
| --- | --- | --- |
| **Component** | **AUC0-12h %** | |
| **Mean** | **SD** |
| M2 | 0.4 | 0.3 |
| M3 | 0.1 | 0.2 |
| M33 | 0.5 | 0.3 |
| M4 | 1.7 | 0.8 |
| M22 | 0.1 | 0.1 |
| M27 | 1.1 | 0.8 |
| M28 | 5.9 | 1.6 |
| M8 | 5.4 | 1.6 |
| M10 | 0.2 | 0.2 |
| M26 | 2.2 | 0.5 |
| M44 | 0.2 | 0.1 |
| M37 | 0.1 | 0.1 |
| M16 | 21.5 | 2.1 |
| M18 | 2.9 | 1.0 |
| M38 | 0.3 | 0.1 |
| M13 | 2.2 | 1.6 |
| **Capmatinib** | **42.9** | **2.9** |
| M20 | 1.5 | 0.2 |
| M49 | 0.1 | 0.1 |
| M21 | 1.3 | 0.2 |
| M50 | 0.3 | 0.1 |
| M19 | 1.0 | 0.2 |
| **Sum of additional components** | 2.2 | 2.6 |
| **Total detected** | 94.1 | 1.9 |
| **Lost during sample processing** | 5.9 | 1.9 |
| **Lost during HPLC** | -- | -- |
| **Total radiolabeled components in original sample** | 100.0 | 0.0 |

AUC0-12h, AUC from 0 to 12 hours; HPLC, high-performance liquid chromatography

**TABLE 2 Capmatinib and metabolites in excreta (means of n=6, SD)**

The total amount of metabolites (% of dose) excreted within 96 hours was estimated from the individual determined excretion data

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Component** | **Excretion 0-96 h (% of dose)** | | | | | |
| **Urine** | | **Feces** | | **Total** | |
| **mean** | **SD** | **mean** | **SD** | **mean** | **SD** |
| M2 | 0.61 | 0.40 | 0.71 | 0.66 | 1.32 | 1.05 |
| M3 | -- | -- | 0.37 | 0.33 | 0.37 | 0.33 |
| M33 | 0.36 | 0.20 | -- | -- | 0.36 | 0.20 |
| M4 | 1.80 | 1.05 | 1.16 | 0.81 | 2.96 | 1.80 |
| M22 | 0.45 | 0.22 | -- | -- | 0.45 | 0.22 |
| M27 | 1.53 | 0.36 | 1.10 | 0.61 | 2.63 | 0.92 |
| M28 | 1.77 | 0.83 | 1.33 | 0.69 | 3.10 | 1.49 |
| M8 | 1.02 | 0.47 | 1.68 | 0.97 | 2.70 | 1.33 |
| M10 | 0.71 | 0.30 | -- | -- | 0.71 | 0.30 |
| M26 | 0.43 | 0.17 | 3.28 | 1.07 | 3.70 | 1.20 |
| M43 | 0.23 | 0.16 | -- | -- | 0.23 | 0.16 |
| M44 | 0.72 | 0.20 | -- | -- | 0.72 | 0.20 |
| M37 | 1.41 | 0.57 | -- | -- | 1.41 | 0.57 |
| M16 | 2.87 | 1.24 | 5.13 | 2.13 | 8.00 | 3.25 |
| M14 | 0.24 | 0.15 | 0.32 | 0.36 | 0.56 | 0.46 |
| M18 | -- | -- | 0.13 | 0.33 | 0.13 | 0.33 |
| M38 | 0.45 | 0.17 | -- | -- | 0.45 | 0.17 |
| M46 | 0.14 | 0.06 | 0.28 | 0.23 | 0.42 | 0.28 |
| M47 | 0.32 | 0.17 | 0.44 | 0.45 | 0.75 | 0.62 |
| M40 | 0.22 | 0.12 | -- | -- | 0.22 | 0.12 |
| M48 | -- | -- | 0.18 | 0.44 | 0.18 | 0.44 |
| M13 | 0.51 | 0.21 | 1.69 | 0.85 | 2.20 | 1.04 |
| **Capmatinib\*** | 0.07 | 0.03 | 42.13 | 23.04 | 42.20 | 23.01 |
| M20 | 0.74 | 0.30 | 2.26 | 1.05 | 3.00 | 1.31 |
| M49 | 0.20 | 0.09 | -- | -- | 0.20 | 0.09 |
| M21 | 1.31 | 0.56 | -- | -- | 1.31 | 0.56 |
| M41 | -- | -- | 1.10 | 0.58 | 1.10 | 0.58 |
| M50 | 0.36 | 0.17 | -- | -- | 0.36 | 0.17 |
| M51 | 0.07 | 0.07 | -- | --- | 0.07 | 0.07 |
| M19 | 0.65 | 0.26 | 1.62 | 0.61 | 2.26 | 0.82 |
| M52 | -- | -- | 1.00 | 0.54 | 1.00 | 0.54 |
| **Sum of additional components** | 2.47 | 1.17 | 4.00 | 2.45 | 6.47 | 3.45 |
| **Total detected** | 21.66 | 8.43 | 69.88 | 10.91 | 91.54 | 5.08 |
| **Lost during sample processing** | 0.00 | 0.00 | 3.89 | 0.99 | 3.89 | 0.99 |
| **Lost during HPLC** | 0.00 | 0.00 | 2.47 | 0.39 | 2.47 | 0.39 |
| **Total analyzed (pool 0-96 h)** | 21.66 | 8.43 | 76.25 | 10.71 | 97.91 | 4.91 |
| **Total excretion (time period 0-168 h)** | 21.82 | 8.48 | 77.85 | 10.25 | 99.67 | 3.25 |

HPLC, high-performance liquid chromatography.

\*Assuming that the drug is stable against intestinal bacterial enzymes, the mean oral absorption of capmatinib (urinary excreted radioactivity: 21.7 ± 8.4% in the analyzed pool 0-96 hours, plus fecal excreted radioactivity: 69.9 ± 10.9% in the analyzed pool 0-96 hours, minus fecal excreted capmatinib: 42.1 ± 23.04%, Table 2) was estimated to be 49.6 ± 20.9% and ranged between 21.0% and 81.7%.

## 2. Determination of capmatinib concentrations in plasma

## Concentration of unchanged capmatinib in plasma was measured by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at WuXi AppTec Co., Ltd. (Shanghai, China). The lower limit of quantification (LLOQ) was 1.00 ng/mL using 0.1 mL of plasma in di-potassium ethylenediaminetetraacetic acid (K2EDTA). For sample preparation, 100 μL aliquot of the samples, 50 μL aliquot of internal standard working solution, and a 300 μL aliquot of 0.5% ammonium hydroxide (NH3∙H2O) in water were added to the designated well of a 96-well plate. After solid phase extraction, the extract was evaporated to dryness. The sample was diluted with 300 μL of 20% acetonitrile [ACN], and analyzed by injecting a 10 μL aliquot of each sample into the LC-MS/MS system in multiple reaction monitoring, positive ion mode using electrospray ionization as the ionization technique (Wuxi AppTec, China).

## The LC-MS/MS system consisted of a CAPCELL PAK MG C18 column (50 × 2 mm, 5 μm particle size) and an API 4000 applied Biosystems/Sciex mass spectrometer. Chromatographic elution was performed using 0.1% formic acid (FA) 1.0 mmol/L ammonium acetate in water (mobile phase A) and 0.1% FA 1.0 mmol/l ammonium acetate in 95% ACN (mobile phase B). The MS transitions observed were m/z 413.1–354.2 for capmatinib and m/z 417.2–382.0 for the internal standard. The LLOQ was 1.0 ng/mL using a 100 µL sample volume.

**Table 3A. Mass spectral data of [14C]capmatinib and metabolites observed in the study**

Data from LC-MS/MS runs of plasma, urine, and feces; metabolites listed in order of elution; electrospray ionization positive ion mode

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Component | Observed ions in LC-MS/MS runs (m/z) | | | | | |
| Fragments | | | | | | Additional major signals |
|  | [M+H]+ | A | A-  CO | B | C |
| M2 | 431 | 414 | 386 | 267 | 158 | 185, 159, 148, 129/130 |
| M3  (CNN019) | 445 | 414 | 386 | 267 | 158 | 185, 159, 148, 129/130 |
| M33 | 591 |  |  | 237a) | 142a) | 415 [M+H –C6H8O6]+, 413 [M+H –C6H10O6]+, 279, 130, 108 |
| M4 | 415 | 398 | 370 |  |  | 258, 231, 224, 214, 158, 130 |
| M22 | 605 | 398a) | 370a) | 251a) |  | 429 [M+H –C6H8O6]+, 411 [M+H –C6H8O6-H2O]+, 148 |
| M27 | 402 |  |  |  |  | 385, 260, 237, 181, 143, 142, 130, 108 |
| M28 | 415 | 398 | 370 | 251 |  | 223, 169, 148, 143, 142 |
| M8  (CMN290) | 429 | 398 | 370 |  | 158 | 272, 214, 187, 130 |
| M10 | 447 |  |  |  |  | 429 [M+H -H2O]+, 398b), 370b), 343, 284, 272, 182, 158b), 130, 106 |
| M26 | 415 |  |  |  |  | 273, 237, 181, 143, 142, 130, 108 |
| M43 | 445 | 414 |  |  |  | 427 [M+H -H2O]+, 400, 370, 353, 352, 240, 222, 214, 174, 156, 130 |
| M44 | 418 |  |  | 253 |  | 400 [M+H -H2O]+, 384, 236, 235, 221, 207, 181, 108 |
| M37 | 431 |  |  | 253 |  | 413 [M+H -H2O]+, 236, 235, 207, 181 |
| M16  (CMN288) | 429 | 398 | 370 | 251 | 142 | 223, 203, 169, 148, 143 |
| M14 | 445 | 414 | 386 |  |  | 427 [M+H -H2O]+, 400, 369, 368, 272, 245, 214, 187, 174, 146, |
| M18  (CNJ294) | 399 | 382 | 354 |  | 142 | 327, 302, 235, 208, 181, 169, 166/167, 154, 143, 142, |
| M38 | 445 | 414 |  | 267 |  | 427 [M+H -H2O]+, 400, 370, 342, 288, 250, 249, 221, 185, 148, 142, 122 |
| M46 | 431 |  |  | 253 | 158 | 414, 274, 235, 146, 108 |
| M47 | 431 | 414 | 386 | 267 | 158 | 358, 304, 249, 203, 185, 148 |
| M40 | 415 | 398 | 370 |  | 142c) | 398, 371, 354, 327, 301, 249, 224, 222, 206, 181, 180, 166, 106 |
| M48 | 429 | 398 | 370 | 251 | 158 | 378, 343, 322, 224, 197, 183, 182, 159, 146, 130 |
| INC280 | 413 | 382 | 354 | 235 | 142 | 327, 214, 208, 181, 169, 166/167, 154, 143 |
| M13  (CMC583) | 400 | 382 | 354 | 235 | 142 | 373, 208, 181, 169, 166/167, 154, 143 |
| M20 | 445 | 414 | 386 | 267 | 158 | 427 [M+H -H2O]+, 358, 265, 249, 203, 148 |
| M49 | 445 | 414 | 386 | 267 | 158 | 427 [M+H -H2O]+, 358, 300, 197, 179, 148, 146, 130, 122 |
| M21  (CJH800) | 429 | 398 | 354c) |  | 142 | 411 [M+H -H2O]+, 384, 327, 249, 207, 206, 181, 180, 166, 106 |
| M41 | 429 | 398 | 370 | 251 | 158 | 343, 315, 214, 197, 183, 182, 159 |
| M50 | 416 |  |  |  |  | 398 [M+H -H2O ]+, 372, 354, 327, 249, 224, 207, 206, 181, 180, 166, 142, 106 |
| M51 | 415 | 398 | 370 |  | 158 | 343, 251, 233, 222, 214 |
| M19  (CJK530) | 429 | 398 | 370 |  | 158 | 411 [M+H -H2O ]+, 343, 251, 233, 222, 214 |
| M52 | 416 | 398 | 370 | 251 | 158 | 343, 233, 222, 214, 197, 183, 182 |
| a) Fragment was formed after neutral loss of anhydroglucuronic acid (C6H8O6)  b) Fragment was formed after neutral loss of water  c) Fragment was formed after loss of oxygen  Nomenclature of fragment ions (Capmatinib as example)  C:\Users\KAMARAA1\AppData\Local\Temp\1\7zO42E82F14\bottom fig of Table3A.tif | | | | | | |

**Table 3B. Exact mass measurements and hydrogen/deuterium exchange**

Data from LC-MS/MS runs of plasma, urine, and feces extract; metabolites listed in order of elution; electrospray ionization positive ion mode.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Component | Proposed formula of [M+H]+ | Nominal mass of [M+H]+ | Rt  [min] | Difference of measured minus calculated mass of [M+H]+ [mDa] | Mass shift of [M+H]+ after H/D exchange  [Da] |
| M2 | C22H16N6O3F | 431 | 15.7 | +0.1 | +5 |
| M3 (CNN019) | C23H18N6O3F | 445 | 20.9 | +/-0.0 | +4 |
| M33 | C29H28N6O7F | 591 | 21.9 | +0.8 | +6 |
| M4 | C22H16N6O2F | 415 | 22.9 | +/-0.0 | +4 |
| M22 | C29H26N6O8F | 605 | 24.4 | +0.9 | +6 |
| M27 | C22H17N5O2F | 402 | 27.2 | +0.1 | +3 |
| M28 | C22H16N6O2F | 415 | 28.4 | -0.1 | +4 |
| M8 (CMN290) | C23H18N6O2F | 429 | 28.7 | +0.4 | +3 |
| M10 | C23H20N6O3F | 447 | 30.1 | +0.6 | +4 |
| M26 | C23H20N6OF | 415 | 31.4 | +0.6 | +3 |
| M43 | C23H18N6O3F | 445 | 32.5 | +0.1 | +3 |
| M44 | C22H17N5O3F | 418 | 33.5 | +0.4 | +3 |
| M37 | C23H20N6O2F | 431 | 35.4 | -0.2 | +3 |
| M16 (CMN288) | C23H18N6O2F | 429 | 36.2 | +0.2 | +3 |
| M14 | C23H18N6O3F | 445 | 36.7 | -0.3 | +4 |
| M18 (CNJ294) | C22H16N6OF | 399 | 38.8 | +0.4 | +3 |
| M38 | C23H18N6O3F | 445 | 42.0 | +/-0.0 | +3 |
| M46 | C23H20N6O2F | 431 | 44.3 | +0.9 | +4 |
| M47 | C22H16N6O3F | 431 | 44.5 | +0.9 | +5 |
| M40 | C22H16N6O2F | 415 | 46.1 | -0.4 | +3 |
| M48 | C23H18N6O2F | 429 | 46.2 | +0.1 | +3 |
| INC280 | C23H18N6OF | 413 | 47.3 | +0.4 | +2 |
| M13 (CMC583) | C22H15N5O2F | 400 | 47.7 | -0.3 | +2 |
| M20 | C23H18N6O3F | 445 | 51.4 | +2.2 | +4 |
| M49 | C23H18N6O3F | 445 | 51.5 | +1.9 | +3 |
| M21 (CJH800) | C23H18N6O2F | 429 | 53.3 | +0.1 | +2 |
| M41 | C23H18N6O2F | 429 | 53.7 | +0.1 | +3 |
| M50 | C22H15N5O3F | 416 | 55.0 | -0.5 | +3 |
| M51 | C22H16N6O2F | 415 | 56.8 | -0.2 | +4 |
| M19 (CJK530) | C23H18N6O2F | 429 | 60.6 | +0.1 | +3 |
| M52 | C22H15N5O3F | 416 | 61.0 | +0.1 | +3 |

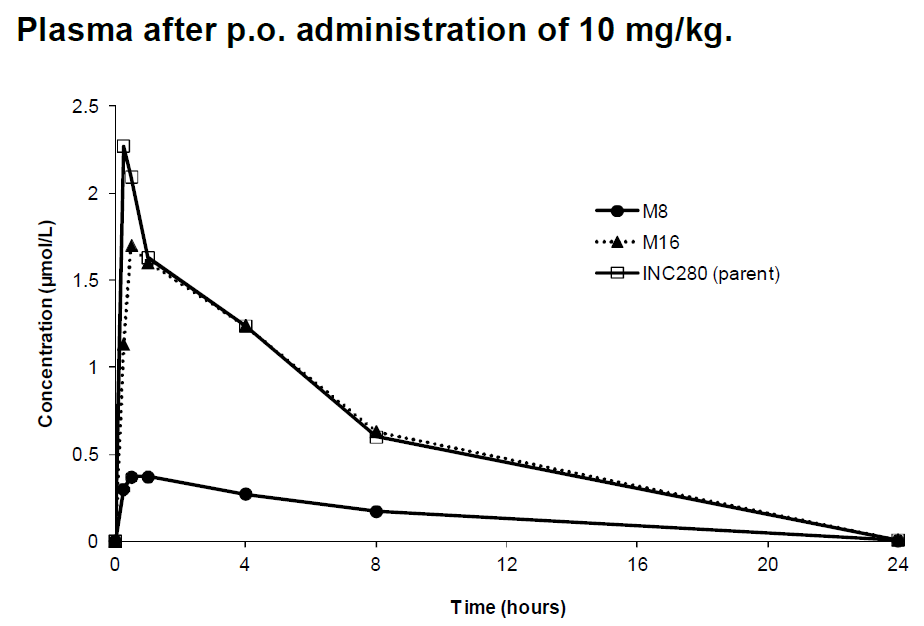
## 3. Metabolism and kinetics in rats after oral administration of capmatinib

**Fig. 1. Plasma metabolite profiles and kinetics of [14C]capmatinib in rats after oral administration**

A) Patterns of metabolites in extracts of pooled plasma samples taken from three rats following oral administration of [14C]capmatinib dihydrochloride. An aliquot was injected into the HPLC column with radioactivity detection.

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B) Concentration of [14C]capmatinib and its main metabolites in plasma of rats following oral administration of [14C]capmatinib dihydrochloride; semi-quantitative data from HPLC analysis with radioactivity detection.



## 4. In vitro assessments:

**Methods:**

**Incubation of [14C]capmatinib with enzymes and tissue fractions**

The incubations were carried out in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C. Typical incubations were prepared as follows: 10 μL of 100 mM MgCl2 (5 mM final concentration), human liver cytosols (HLC), enzymes or tissue fractions, and 20 μL NADPH (if needed, 1 mM final concentration) were added to an appropriate volume of the buffer and pre-incubated for 3 min at 37°C. The reaction was started by addition of the substrate. The final concentrations of organic solvent were maximally 0.5% (v/v). The samples were incubated at 37°C in a thermomixer (Eppendorf 5355 comfort) with agitation at 500 rpm. In the HLC kinetics assay, aldehyde oxidase (AO)/xanthine oxidase (XO) mapping, and in the chemical inhibition assays, the reaction was started by addition of HLC. The incubations with recombinant human (rh) AO of Cypex were carried out in 25 mM potassium buffer, pH 7.4, containing 1 mM EDTA, and the incubation with XO from buttermilk was carried out in 50 mM TRIS buffer, pH 7.5, at 25°C and 37°C. The *Escherichia coli* cells expressing AO were provided as a suspension in 100 mM di-glycine buffer, pH 7.4, and the incubation was carried out in this solution at 30°C. The enzymatic reaction was stopped, and the protein was precipitated by addition of an equal volume of acetonitrile. After 30 min at −80°C (or overnight at −20°C), the samples were centrifuged at 30,000*g* for 15 min and the supernatant was withdrawn. Aliquots were analyzed by liquid scintillation counting, and the supernatants were diluted with water and acetonitrile to achieve a final solution containing less than 5 or 12% of the organic solvent. Samples were analyzed by high-performance liquid chromatography (HPLC) combined with radiodetection.

**Concentration dependent biotransformation of [14C]capmatinib in HLC**

Enzyme kinetic parameters Km and Vmax were determined by incubating capmatinib with pooled HLC (0.4 mg/mL) at 15 nominal substrate concentrations ranging from 0.5 to 300 μM for 120 min. The enzyme kinetics was performed using the established linear conditions (time, enzyme concentration), and less than 20% of the initial substrate was consumed at the end of incubations. Experimental data (rates of formation of metabolites M16 and M19) were analyzed by nonlinear regression analysis considering different kinetic models (Michaelis-Menten, Hill, isoenzyme, substrate inhibition) as provided by the Enzyme Kinetics module, SigmaPlot.

**Analysis of enzyme kinetics**

The enzyme kinetics of the biotransformation of [14C]capmatinib in HLC was investigated in incubations performed under linear conditions with respect to time (2 h) and protein content (0.4 mg/mL). Enzyme kinetic parameters Vmax and Km of the biotransformation by HLC were calculated by using SigmaPlot Version 12.1. Enzyme Kinetics module Version 1.3 software (SPSS Science Inc., Chicago, IL, USA). The intrinsic clearance (CLint) was calculated by the equation: CLint = Vmax/Km. IC50 values were estimated by graphic extrapolation

**Biotransformation of [14C]capmatinib by purified or recombinant molybdenum hydroxylases**

Purified or recombinant enzymes were used to screen the involvement of aldehyde and XO enzymes in the biotransformation of capmatinib. Incubation experiments with a panel of six molybdenum hydroxylases, rh AO (whole *E. coli* cells suspension), rh AO (cytosolic extract of *E. coli*), rh AO (extract of HEK293 cells), XO from buttermilk and XO microbial, and rh XDH, were conducted with 5 μM and 15 μM of [14C]capmatinib.

**Inhibition of [14C]capmatinib biotransformation by chemical inhibitors**

The biotransformation of capmatinib was tested at 8 μM substrate concentrations in the presence of four AO inhibitors (raloxifene, menadione, isovanillin, and hydralazine) and one XO inhibitor (allopurinol).

**Molybdenum hydroxylases phenotyping of [14C]capmatinib: involvement of AO and XO in the metabolic formation of M16 and M19**

Molybdenum containing hydroxylases (AO/XO) use water (H2O) in their catalytic mechanisms of oxidation (Garattin, 2012)) rather than molecular oxygen (O2), as it is the case with the cytochrome P450 oxidases (Guengerich, 2001). Based on the complex biotransformation pathways, AO may contribute to the formation of the major human metabolites M4, M5, M16, and M19 (in vivo and/or in vitro).

A mechanistic experiment using 18O-water (Dalvie et al., 2010; Diamond et al., 2010; Hutzler et al., 2012) was performed to check the contribution of molybdenum-containing hydroxylases in the biotransformation of capmatinib in a preliminary experimental set-up. A pool of cryopreserved human hepatocytes prepared from 20 mixed (male/female) individual donors was obtained from Celsis In Vitro Technologies Inc. (Baltimore, MD, USA, catalog No X008000, lot SSB).

Incubations of [14C]capmatinib with human hepatocytes in the presence of 18O-water were performed to obtain information about the potential involvement of molybdenum-containing hydroxylases in the formation of metabolites M16 and M19. In addition, a positive control (O6-Benzylguanine) was included to check the AO-activity of the hepatocytes. AO plays an important role in Phase I metabolism. It catalyzes the oxidation of a carbon adjacent to a nitrogen in a N-heterocycle. Incubates were analyzed by liquid chromatography-mass spectrometry (LC-MS).

**RESULTS**

**Concentration dependent biotransformation of [14C] capmatinib in HLC**

By plotting the experimental data as Michaelis-Menten plot, the kinetic data were best fitted with the substrate inhibition model for both metabolite formations. Non-linear regression analysis of the rate of metabolism versus substrate concentration revealed an apparent kinetic constant Km of 8.84 ± 2.01 μM, Vmax of 21.5 ± 2.6 pmol/min/mg, and Ki of 40.1 ± 8.4 μM for the formation of metabolite M16. For the formation of metabolite M19, Km of 18.5 ± 6.4 μM, Vmax of 11.2 ± 2.4 pmol/min/mg, and Ki of 54.1 ± 18.2 μM were calculated. The derived intrinsic clearances (Vmax/Km) of the hepatic metabolism of capmatinib were low (2.43 μL/mg/min and 0.605 μL/mg/min for M16 and M19 formation, respectively).

**TABLE 4A Concentration-dependent biotransformation of [14C] Capmatinib to M16**

Individual and mean values of 2 incubations are given

|  |  |  |  |
| --- | --- | --- | --- |
| **Mean**  **[14C] Capmatinib**  **concentration (μM)** | **Rate of M16 formation**  **(pmol/min/mg protein)** | | |
| **Sample A** | **Sample B** | **Average** |
| 0.422 | 1.04 | 1.04 | 1.04 |
| 1.12 | 2.41 | 2.27 | 2.34 |
| 2.51 | 5.33 | 5.23 | 5.28 |
| 4.65 | 7.86 | 6.35 | 7.11 |
| 6.75 | 8.27 | 7.83 | 8.05 |
| 10.7 | 10.3 | 9.80 | 10.0 |
| 15.4 | 11.8 | 11.8 | 11.8 |
| 19.9 | 9.32 | 13.2 | 11.2 |
| 26.4 | 9.47 | 10.6 | 10.1 |
| 46.9 | 10.2 | 8.60 | 9.41 |
| 76.1 | 7.77 | 7.18 | 7.48 |
| 102 | 5.81 | 6.54 | 6.18 |
| 149 | 3.75 | 3.69 | 3.72 |
| 202 | 3.06 | 2.65 | 2.86 |
| 336 | 2.91 | 3.65 | 3.28 |

**TABLE 4B Concentration-dependent biotransformation of [14C] Capmatinib to M19**

Individual and mean values of 2 incubations are provided

|  |  |  |  |
| --- | --- | --- | --- |
| **Mean**  **[14C] Capmatinib**  **concentration (μM)** | **Rate of M19 formation**  **(pmol/min/mg protein)** | | |
| **Sample A** | **Sample B** | **Average** |
| 0.422 | 0.316 | 0.288 | 0.302 |
| 1.12 | 0.722 | 0.616 | 0.669 |
| 2.51 | 1.48 | 1.57 | 1.53 |
| 4.65 | 2.42 | 2.03 | 2.23 |
| 6.75 | 3.01 | 3.12 | 3.06 |
| 10.7 | 3.55 | 3.57 | 3.56 |
| 15.4 | 4.08 | 4.70 | 4.39 |
| 19.9 | 3.89 | 5.75 | 4.82 |
| 26.4 | 5.08 | 5.41 | 5.25 |
| 46.9 | 6.33 | 4.85 | 5.59 |
| 76.1 | 3.72 | 4.18 | 3.95 |
| 102 | 3.23 | 3.38 | 3.31 |
| 149 | 2.50 | 3.02 | 2.76 |
| 202 | 1.75 | 2.21 | 1.98 |
| 336 | 2.18 | 2.92 | 2.55 |

**Analysis of enzyme kinetics**

The enzyme kinetic parameters for the formation of M16 and M19 by AO, XO, and HLC were estimated by simulation assuming Michaelis-Menten type behavior for the two substrate concentrations (5 μM and 15 μM). Since the expression levels of the different molybdenum hydroxylases or AO isoforms are not well established quantitatively like hepatic CYPs, their activities could not be expressed in relation to the amount of AO protein in the assay, and the catalytic efficiency of the different isoforms might not reflect the respective contribution ratio of each isoform to capmatinib metabolism in vivo.

**TABLE 5 Estimation of enzyme kinetics constants**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Enzyme** | **Formation rate**  **V1**  **(pmol/min/mg)** | **Formation rate**  **V2**  **(pmol/min/mg)** | **Capmatinib**  **conc.**  **S1**  **(μM)** | **Capmatinib**  **conc.**  **S2**  **(μM)** | **Vmax**  **(pmol/min/mg)** | **Km**  **(μM)** | **(Vm/Km)**  **(μl/min/mg)** | **Metabolite** |
| **rh AO (Cypex)** | 0.328 | 0.438 | 5 | 15 | 0.525 | 3.00 | 0.175 | M16 + M19 |
| **rh AO in**  ***E. coli* cells (Novartis)** | 0.0023 | 0.0088 | 5 | 15 | n.a | n.a |  | M16 + M19 |
| **XO (microbial)** | 4.01 | 9.92 | 5 | 15 | 38.0 | 42.4 | 0.896 | M19 |
| **HLC** | 7.27 | 11.88 | 5 | 15 | 17.4 | 6.97 | 2.50 | M16 |
| **HLC** | 2.11 | 3.84 | 5 | 15 | 6.53 | 10.5 | 0.623 | M19 |
| **HLC** | 9.38 | 15.72 | 5 | 15 | 23.8 | 7.67 | 3.097 | M16 + M19 |

AO, aldehyde oxidase; HLC, human liver cytosol; n.a., not applicable (concentration of AO enzymes is unknown); rh, recombinant human .

**Biotransformation of [14C]capmatinib by purified or recombinant molybdenum hydroxylases**

Significant amount of the metabolite M16 were found with AO incubation (whole *E. coli* cells and cytosolic extract of *E. coli*), while only traces or none was detected with other enzymes. Metabolite M19 was significantly observed with XO (microbial) and AO incubations (whole *E. coli* cells), while only traces or none was detected with other enzymes. The biotransformation rates in the incubates of [14C]capmatinib (5 μM and 15 μM initial concentration) with AO, XO, and XDH are listed in Table 6A and Table 6B.

**TABLE 6A. Phenotyping with molybdenum hydroxylases for the formation of M16**

Incubations of rh AO (Origene), XO (microbial), rh XDH and XO (microbial) were carried out in 100 mM potassium phosphate, pH 7.4.

Incubations of rh AO (Cypex) were carried out in 25 mM potassium phosphate buffer (pH 7.4 containing 1 mM EDTA) and incubation with XO (buttermilk) in 50 mM TRIS buffer (pH 7.5). The *E. coli* cells expressing AO were provided as suspension in 100 mM di-glycine buffer pH 7.4 for the incubation. The protein concentration was 0.4 mg/mL except for rh AO and rh XDH of Origene (0.05 mg/mL) and for the *E. coli* cells expressing AO (wet biomass concentration was 148 mg/mL and 144 mg/mL, for 5 μM and 15 μM INC280, respectively).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Enzymes and condition** | **Metabolite M16 formation** | | | |
| **5 μM**  **Capmatinib** | | **15 μM**  **Capmatinib** | |
| **Radioactivity (%)** | **Formation rate (pmol/min/mg protein** | **Radioactivity (%)** | **Formation rate (pmol/min/mg protein** |
| **Incubation without protein** | 0 | 0 | 0 | 0 |
| **HL cytosol incubated 2 h at 37o C** | 6.98 | 7.27 | 3.80 | 11.9 |
| **rh AO, *E. coli* cells incubated 6 h at 30o C** | 1.72 | 0.0016a | 2.16 | 0.0062a |
| **rh AO incubated 2 h at 37o C (Supplier Cypex)** | 0.28 | 0.292 | 0.09 | 0.266 |
| **rh AO incubated 2 h at 37o C (Supplier Origene)** | 0.04 | 0.292 | 0.05 | 1.25 |
| **rh XDH incubated 2 h at 37o C** | 0.01 | 0.042 | 0.04 | 1.00 |
| **XO (microbial) incubated 2 h at 37o C** | 0.01 | 0.010 | n.d. | n.d. |
| **XO (buttermilk) incubated 2 h at 37o C** | 0.01 | 0.010 | 0.03 | 0.094 |
| **XO (buttermilk) incubated 2 h at 37o C** | n.d. | n.d. | 0.01 | 0.016 |

AO, aldehyde oxidase; HL, human liver; n.d., not detected; rh, recombinant human; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

apmol/min/mg wet biomass of *E. coli* cells

**TABLE 6B Phenotyping with molybdenum hydroxylases for the formation of M19**

Incubations of rh AO (Origene), XO (microbial), rh XDH and XO (microbial) were carried out in 100 mM potassium phosphate, pH 7.4.

Incubations of rh AO (Cypex) were carried out in 25 mM potassium phosphate buffer (pH 7.4 containing 1 mM EDTA) and incubation with XO (buttermilk) in 50 mM TRIS buffer (pH 7.5). The *E. coli* cells expressing AO were provided as suspension in 100 mM di-glycine buffer pH7.4 and the incubation was carried out in this suspension. The protein concentration was 0.4 mg/mL except for rh AO and rh XDH of Origene (0.05 mg/mL) and for the *E. coli* cells expressing AO (wet biomass concentration was 148 mg/mL and 144 mg/mL, for 5 μM and 15 μM INC280, respectively).

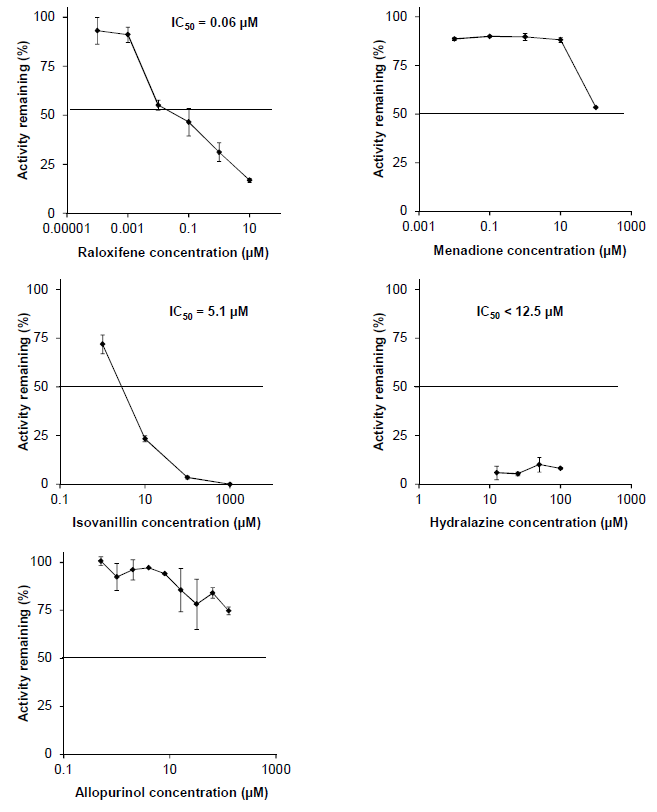
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Enzymes and condition** | **Metabolite M19 formation** | | | |
| **5 μM**  **Capmatinib** | | **5 μM**  **Capmatinib** | |
| **Radioactivity (%)** | **Formation rate (pmol/min/mg protein** | **Radioactivity (%)** | **Formation rate (pmol/min/mg protein** |
| **Incubation without protein** | 0 | 0 | 0 | 0 |
| **HL cytosol incubated 2 h at 37o C** | 2.03 | 2.11 | 1.23 | 3.84 |
| **rh AO, *E. coli* cells incubated 6 h at 30 o C** | 0.73 | 0.00069a | 0.87 | 0.0025 |
| **rh AO incubated 2 h at 37o C (Supplier Cypex)** | 0.04 | 0.036 | 0.06 | 0.172 |
| **rh AO incubated 2 h at 37o C (Supplier Origene)** | 0.01 | 0.083 | 0.04 | 0.875 |
| **rh XDH incubated 2 h at 37o C** | 0.02 | 0.167 | n.d. | n.d. |
| **XO (microbial) incubated 2 h at 37o C** | 3.85 | 4.01 | 3.18 | 9.92 |
| **XO (buttermilk) incubated 2 h at 37o C** | 0.01 | 0.010 | 0.03 | 0.094 |
| **XO (buttermilk) incubated 2 h at 37o C** | 0.02 | 0.016 | 0.01 | 0.031 |

AO, aldehyde oxidase; HL, human liver; rh, recombinant human; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

apmol/min/mg wet biomass of *E. coli* cells.

**Inhibition of [14C]capmatinib biotransformation by chemical inhibitors**

All the AO inhibitors showed total or strong inhibition (71–100%) on M16 and M19 formations (except for menadione where the maximal inhibition on M19 formation was 47%). Inhibition by AO inhibitors were generally stronger (almost complete) to M16 formation than to M19 formation (most partial). The XO inhibitor allopurinol showed lower and partial inhibition to the formation of M16 and M19 (25–26%).

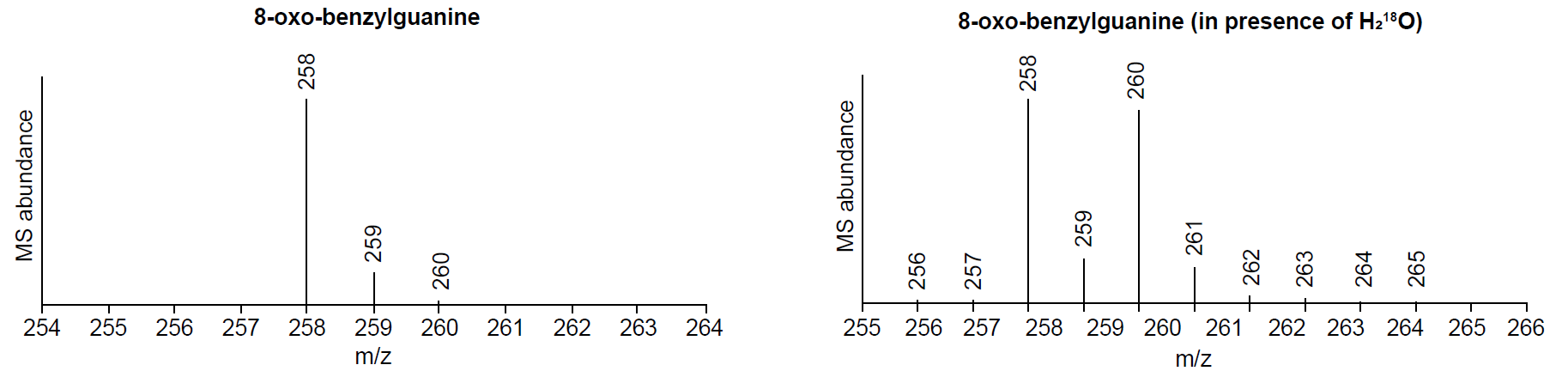
**Fig. 2. Effect of chemical inhibitors on capmatinib metabolism in HLC (M19 formation)**

**Incubation of [14C]capmatinib with human hepatocytes in the presence and absence of O18-water**

Incubation of [14C]capmatinib with human hepatocytes in the presence of 18O-water showed incorporation of 18O into the metabolites M16 and M19 (41% and 33%, respectively). Isotope patterns of the metabolites M16 and M19 with molecule ions of the 14C-labelled [M+H]+ at m/z 431 (+16 Da) and m/z 433 (+18 Da) were obtained.

**Incubations of O6-Benzylguanine with human hepatocytes in the absence and presence of 18O-water**

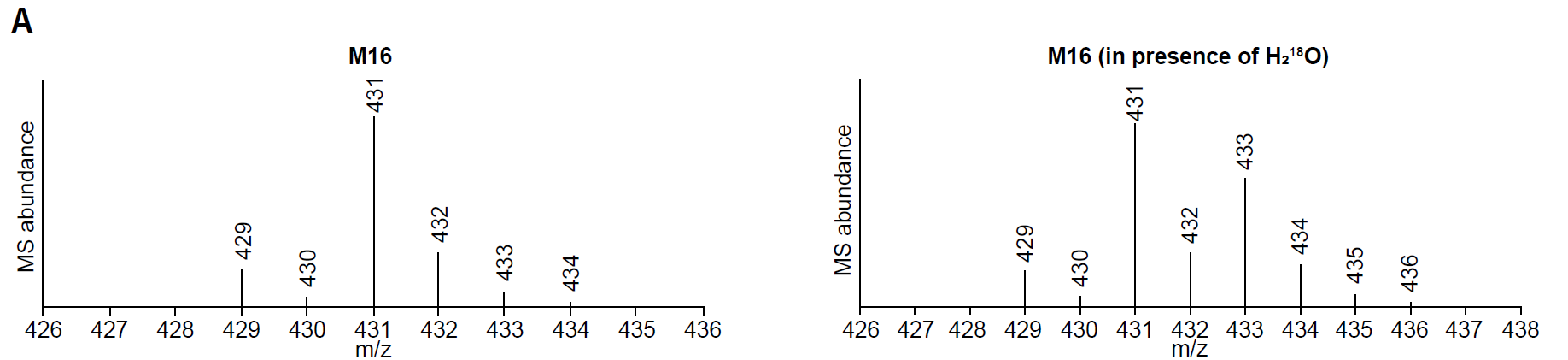
**Fig. 3.**



Absolute incorporation of 18O: 46 %

**Incubation of [14C] capmatinib with human hepatocytes in the absence and presence of 18O-water**

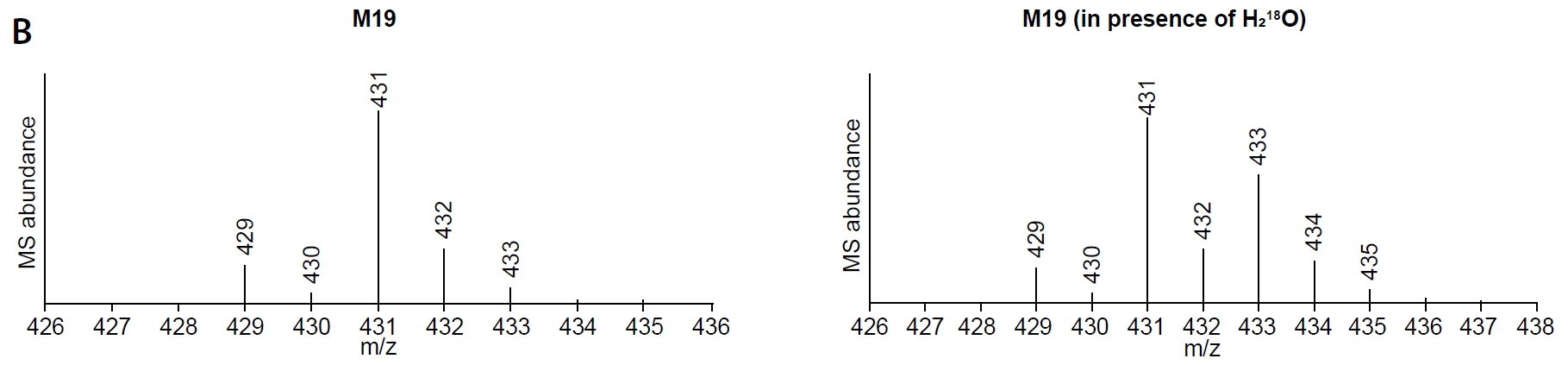
**Fig. 4A.**



Absolute incorporation of 18O: 41 %

Relative incorporation of 18O: 88 %

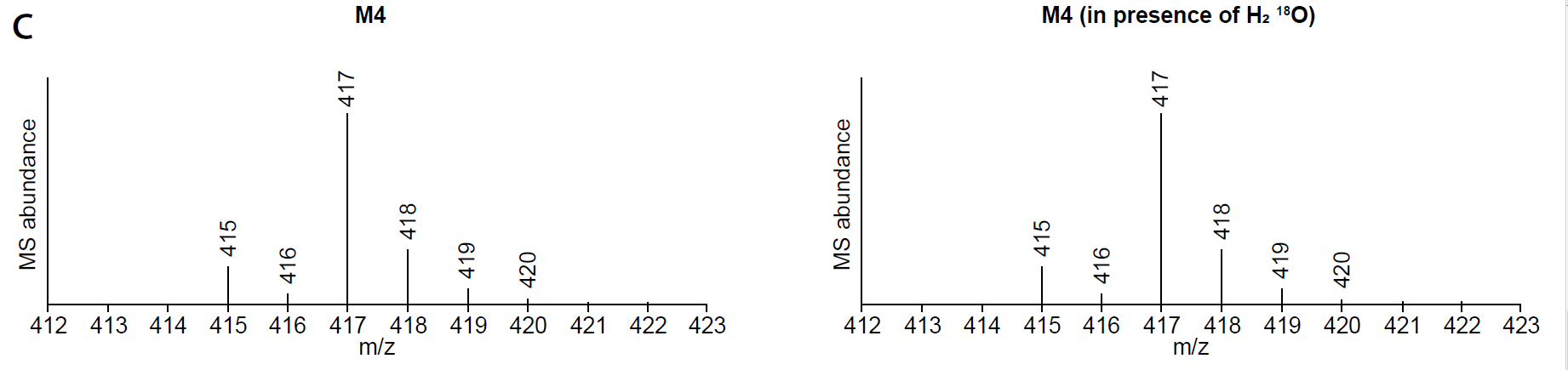
**Fig. 4B.**



Absolute incorporation of 18O: 33 %

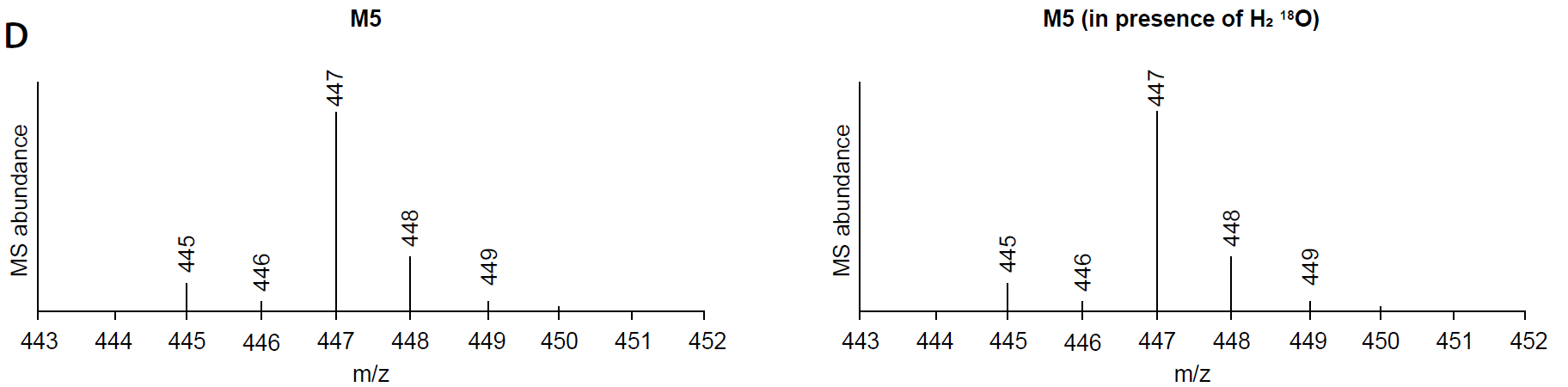
Relative incorporation of 18O: 72 %

**Fig. 4C.**



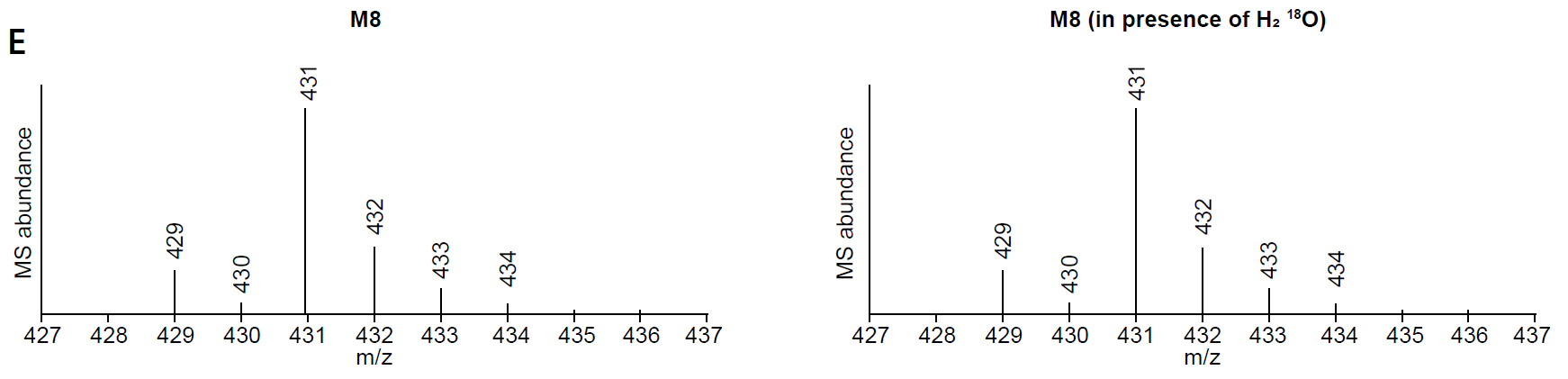
No incorporation of 18O

**Fig. 4D.**



No incorporation of 18O

**Fig. 4E.**



No incorporation of 18O

**Identification of molybdenum hydroxylases-mediated metabolites of capmatinib in HμREL human pooled primary hepatic co-culture in the absence and presence of 18O‑water**

Molybdenum-containing hydroxylases (AO/XO) use water in their catalytic mechanism of oxidation (Garattini and Terao, 2012) rather than molecular oxygen, as is the case with the cytochrome P450 oxidase (Guengerich, 2001). Incubations of [14C]capmatinib with Hμrel human pooled primary hepatic co-culture in the presence of 18O-water were performed to assess the potential involvement of molybdenum-containing hydroxylases in the formation of its metabolites (Hutzler et al, 2012). In addition, a positive control (O6-benzylguanine) was included to check the AO-activity of the hepatic co-culture. Incubates were analyzed by LC-MS.

After incorporation of 18O into the metabolite, a shift of two mass units of the [M+H]+ could be observed. By using same volumes of medium and 18O water (containing 97% 18O) for the incubations, the maximal possible percentage of incorporation of 18O was 48.5%. In this study, the part of medium was slightly higher than the 18O-water, because 20-50 μL medium had to be left in each vial before adding the same amounts of the medium and 18O-water. Therefore, the maximal percentage of incorporation was less than 48.5%.

**TABLE 7 Summary of 18O incorporation into metabolites of capmatinib and the positive control O6-benzylguanine in incubations with Hμrel® human pooled primary hepatic co-culture and human hepatocytes**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Compound** | **Culture** | **Time point (h)** | **Incorporation 18O (%)** | **Involvement of AO/XO in the formation of metabolites** |
| **8-oxo-benzylguaninea** | Hepatocytes | 2 | 46b | 100 |
| Hµrel | 2 | 40c | 100 |
| **M2** | Hepatocytes | 6 | 33 | 72 |
| **M3** | Hepatocytes | 6 | 39 | 85 |
| **M28** | Hepatocytes | 6 | 30 | 65 |
| **M10** | Hepatocytes | 6 | 28 | 61 |
| Hµrel | 48 | 27 | 68 |
| **M16** | Hepatocytes | 6 | 41 | 89 |
| Hµrel | 48 | 32 | 80 |
| **M14** | Hepatocytes | 6 | 36 | 78 |
| **M38** | Hµrel | 168 | 26 | 65 |
| **M20d** | Hepatocytes | 6 | 49/19 | 107/83 |
| **M49** | Hepatocytes | 6 | 35 | 76 |
| **M19** | Hepatocytes | 6 | 33 | 72 |
| Hµrel | 48 | 24 | 60 |

aMetabolite of positive control O6-benzylguanine.

bThe theoretical maximal percentage of incorporation of 18O was 48.5%.

cThe theoretical maximal percentage of incorporation of 18O was 48.5%. A higher part of medium compared to the 18O-water was used in the experiments therefore, the maximal incorporation was lower.

dTwo 18O are incorporated into the metabolite.

AO, aldehyde oxidase; XO, xanthine oxidase.

**In vitro metabolism of [14C]capmatinib in mouse, rat, dog, monkey, and human hepatocytes and in HLM**

[14C]capmatinib was incubated with hepatocytes from male ICR/CD-1 mice, male Sprague-Dawley rats, male beagle dogs, male cynomolgus monkeys and humans (mixed males and females) at 10 μM concentrations for up to 24 h. Incubations of 5 μM [14C]capmatinib with HLM for up to 0.5 h were also performed. The incubate profiles were analyzed by HPLC with radioactivity detection. Metabolite structures were characterized by LC-MS**.** The following scheme describes tentatively the biotransformation of [14C]capmatinib observed in vitro using mouse, rat, dog, cynomolgus monkey, human hepatocytes, and human microsomal incubations.

**Fig. 5. Proposed metabolic pathway of capmatinib**

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Radiolabeled components in incubations of [14C]INC280 with hepatocytes of mouse, rat, dog,

monkey and human (10 μM, 6 hours) and human microsomal incubation (5 μM, 0.5 h),

proposed structures shown in table 8 The relative proportions of parent compound and

metabolites in the incubations were determined by HPLC with radioactivity detection.

**TABLE 8 Species comparison of metabolites**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Component** | **Proportion of radioactivity [% of total]**  **species** | | | | | |
| **Mouse** | **Rat** | **Dog** | **Monkey** | **Human** | **Human** |
| **Hepatocytes** | | | | | **Microsomes** |
| **P9.32** | - | - | - | 0.63 | 1.67 | - |
| **M1** | - | - | - | - | 0.78 | - |
| **M2** | Tr | - | - | 1.19 | 1.56 | - |
| **M3** | 1.52 | 1.04 | - | 6.89 | 0.80 | - |
| **P20.80** | - | - | - | 1.05 | 1.77 | 0.95 |
| **M4** | 0.70 | Tr | 1.78 a | 3.54 a | 11.34 a | 0.74 a |
| **M5** | Tr | Tr | 3.37 a | 3.20 a | 10.54 a | 4.05 a |
| **M6** | - | Tr | 1.60 | 4.03b | Tr | - |
| **M22** | 0.83 | 2.53 | - | 4.03 b | 1.38 | - |
| **P25.68** | - | - | 5.75 | 2.94 | Tr | - |
| **M23** | - | - | 0.51 | - | - | - |
| **M7** | - | - | 1.76 | - | - | - |
| **M8 c** | 19.23 | 16.24 | 8.37 | 23.97 | 24.05 | 32.31 |
| **M9** | - | - | 1.94 | - |  | - |
| **M10** | - | - | - | - | 1.72 | - |
| **M24** | 1.11 | 0.42 | 4.14 | 4.49b | 1.73 | 1.34 |
| **M25** | Tr | 0.64 | - | 0.70b | Tr | - |
| **M11** | - | - | 1.93 | - | - | - |
| **M26** | - | - | - | - | 1.31 | - |
| **M12** | - | - | 0.1 | - | - | - |
| **M14** | 2.13 | Tr | Tr | 1.79 | 1.68 | - |
| **M15** | - |  | 0.62b |  |  | - |
| **M16** | 21.56 | 25.96 | 1.55 b | 10.03 | 4.35 | Tr |
| **M17** | - | - | - | - | 1.95 | 5.39 |
| **M18** | - | - | - | - | 3.17 | 0.73 |
| **M13** | 7.99 | 12.19 | 49.26 | 1.90 | 0.65 | Tr |
| **M20** | 3.28 | 1.47 | - | 1.26 | 0.38 |  |
| **M21** | 3.19 | 0.81 | 2.60 | 5.06 | 2.31 | 7.04 |
| **M19** | 3.68 | 2.32 | - | 1.50 | 1.67 | 0.70 |
| **Capmatinib** | 23.13 | 27.55 | 0.64 | 2.40 | 12.87 | 40.97 |
| **Othersd** | 11.65 | 8.83 | 14.08 | 19.40 | 12.32 | 5.78 |

aIn case of human hepatocyte incubation, M4 and M5 were separated with different chromatographic conditions. Otherwise, M4 and M5 were estimated from the MS abundance of the respective protonated molecule ions.

bMetabolites were estimated from the MS abundance of the protonated molecule or fragment ions.

cIn a control incubation with HepatoZYMETM, M8 was detected in traces (1.7% after 6 hours after incubation time).

dSum of other radiolabeled components. None of them represented more than 1% in human and 3% in other species of the total radioactivity.

MS, mass spectrometry; Tr, Traces of the metabolite were detected by mass spectrometry.

**Identification of human cytochrome P450 enzymes involved in the oxidative metabolism of capmatinib in human liver microsomes (HLM)**

**Methods**

In vitro incubation of [14C]capmatinib with HLM and recombinant enzymes, chemical inhibition by enzyme-selective inhibitors, enzyme kinetic experiments to determine Km and Vmax, and correlation analysis were conducted. A pool of HLM prepared from 50 individual donors was obtained from BD Biosciences (Woburn, MA, USA, catalog No 457081, lot 82087). Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing human CYP enzymes and insect cell membrane preparations (negative control) were obtained from BD Biosciences (Woburn, MA, USA).

**Biotransformation of [14C]capmatinib with HLM**

Enzyme kinetic parameters Km and Vmax were determined by incubating capmatinib with pooled HLM (0.3 mg/mL) at 12 substrate concentrations ranging from 0.5 to 100 μM for 5 min. The enzyme kinetics were performed using established linear conditions (time, enzyme concentration), and less than 20% of the initial substrate was consumed at the end of incubations. Experimental data (rate of formation of the sum of all metabolites) were analyzed by nonlinear regression analysis considering different kinetic models (Michaelis-Menten, Hill, isoenzyme, substrate inhibition) as provided by the Enzyme Kinetics module, SigmaPlot (S6.3).

**Biotransformation of [14C]capmatinib with rh CYPs and FMO**

Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing one single human cytochrome P450 and FMO isoenzyme were used to assess the involvement of specific enzymes in the biotransformation of [14C]capmatinib. Incubation experiments with a panel of 18 recombinant CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F3B, and CYP4F12) and three recombinant FMOs (FMO1, FMO3, and FMO5) were conducted for each isoenzyme with 5 μM and 75 μM capmatinib and 20 pmol CYP/mL or 0.1 mg FMO/mL. The incubations were carried out in 0.1 M phosphate buffer, pH 7.4, at 37°C. Typical incubations of 200 μL total volume were prepared and the reaction was started by addition of 20 μL of a fresh 10 mM solution of NADPH (1 mM final concentration). The samples were incubated at 37°C in a thermomixer (Eppendorf 5355 comfort) with agitation at 500 rpm. For incubations with CYP2A6, CYP2C9, CYP2C18, and CYP4A11, the phosphate buffer was replaced with TRIS buffer (50 mM, pH 7.5).

**Analysis of enzyme kinetics**

Enzyme kinetic parameters Vmax and Km of the biotransformation by HLM and major metabolizing enzymes were calculated by using SigmaPlot Version 11.0 (S6.3), Enzyme Kinetics module Version 1.3 software (SPSS Science Inc., Chicago, IL, USA). The intrinsic clearance was calculated by the equation: CLint = Vmax/Km. The inter-system extrapolation factors (ISEF) for each CYP enzyme were calculated by the equation:

ISEF = Vmax(HLM)/[Vmax(rhCYP) • abundance]

**Inhibition of [14C]capmatinib biotransformation by chemical inhibitors**

The biotransformation of capmatinib was tested at 10 μM substrate concentration in the presence of nine individual chemical inhibitors (furafylline, montelukast, sulfaphenazole, ticlopidine, quinidine, sodium diethyldithiocarbamate [DETC], ketoconazole, azamulin, and methimazole). The concentration ranges of the inhibitors used were selected to encompass reported apparent Ki values (median and ranges) for inhibition of specific CYP enzymes.

**Correlation analysis in HLM from individual donors**

Correlation analyses were conducted with one set of 16 individual donor liver microsomes to identify and characterize the major metabolizing CYPs or FMOs in HLM. For this aim, [14C]capmatinib (10 μM) metabolism rates in HLM from 16 individuals were correlated with CYP and FMO marker enzyme activities.

**RESULTS**

**Biotransformation of [14C]capmatinib with HLM**

Plotting the experimental data as Michaelis-Menten plot and as Eadie-Hofstee plot, the kinetic data were best fitted with the Hill model. From the equation of this model, the apparent kinetic constants Km of 11.4 ± 1.0 μM, Vmax of 1969 ± 69 pmol/min/mg, and Hill constant of 1.09 ± 0.07 were determined. The derived intrinsic clearance (Vmax/Km) of the hepatic metabolism of capmatinib was 173 μL/mg/min.

**TABLE 9 Enzyme kinetics**

|  |  |  |  |
| --- | --- | --- | --- |
| **Vmax**  **(pmol/min/mg)** | **(μM)** | ***V*max/**  **(μL/mg/min)** | **Hill constant** |
| 1969 ± 69 | 11.4 ± 1.0 | 173 | 1.09 ± 0.07 |

**Biotransformation of [14C]capmatinib with rh CYPs and FMO**

At both concentrations, CYP1A1 and CYP3A4 showed the highest turnover under the experimental conditions used. Low metabolic activities were also observed in incubations with CYP1A2, CYP2D6, and CYP2J2, while only trace or no metabolism was detected with other CYP isoenzymes. Comparing to HLM, no metabolic activities (FMO3 and FMO5) or trace levels (FMO1) were observed in incubations with the recombinant FMO isoenzymes.

**Fig. 6. Biotransformation with recombinant CYP450s and FMOs**

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FMO, Flavin-containing monooxygenase; HLM, Human liver microsomes

**Analysis of enzyme kinetics**

Kinetic constants using the Michaelis-Menten equation fitted with Michaelis-Menten model for CYP1A2 (Km: 26.9 ± 3.0 μM; Vmax: 723 ± 26 pmol/min/nmol), CYP2D6 (Km: 13.0 ± 3.0 μM; Vmax: 902 ± 56 pmol/min/nmol), and CYP3A4 (Km: 3.30 ± 0.44 μM; Vmax: 22116 ± 713 pmol/min/nmol) were determined. The derived intrinsic clearances (Vmax/Km) of capmatinib were 26.9 μL/nmol/min, 69.4 μL/nmol/min, and 6702 μL/nmol/min for CYP1A2, CYP2D6, and CYP3A4, respectively. With regard to their importance in hepatic metabolic clearance of capmatinib, the unbound intrinsic clearance (CLint,u) relative to their abundance in HLM was calculated.

**TABLE 10 Enzyme kinetics**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Enzyme** | **Km**  **(μM)** | **Vmax**  **(1/min)** | **fumic** | **ISEF** | **Vmax/Km**  **µl (min. pmol)** | **ISEF\* Vmax/Km,u**  **µl (min. pmol)** | **Abundance**  **(pmol P450/mg)** | **Rel.**  **abundance** | **Rel. Clint,u in HLM**  **µl(min.mg)** | **fm, CYP**  **(%)** |
| **CYP1A2** | 26.9 | 0.723 | 0.79 | 0.385 | 0.027 | 0.013 | 52 | 10.2% | 0.68 | 0.30 |
| **CYP2A6** |  |  |  |  |  |  | 20 | 3.9% |  |  |
| **CYP2B6** |  |  |  |  |  |  | 17 | 3.3% |  |  |
| **CYP2C8** |  |  |  |  |  |  | 24 | 4.7% |  |  |
| **CYP2C9** |  |  |  |  |  |  | 73 | 14.3% |  |  |
| **CYP2C18** |  |  |  |  |  |  | 1 | 0.2% |  |  |
| **CYP2C19** |  |  |  |  |  |  | 14 | 2.7% |  |  |
| **CYP2D6** | 13 | 0.902 | 0.74 | 0.224 | 0.069 | 0.021 | 8 | 1.6% | 0.17 | 0.07 |
| **CYP2E1** |  |  |  |  |  |  | 61 | 11.9% |  |  |
| **CYP 2J2** | 11.8 | 1.04 | 0.84 | 0.98 | 0.088 | 0.103 | 1.2 | 0.2% | 0.1 | 0.05 |
| **CYP 3A4** | 3.3 | 22.12 | 0.82 | 0.202 | 6.702 | 1.651 | 137 | 26.8% | 226.2 | 99.6 |
| **CYP3A5** |  |  |  |  |  |  | 103 | 20.1% |  |  |
| **Total CYP** |  |  |  |  |  |  | 511 | 100% | 227.2 | 100 |

**Inhibition of [14C]capmatinib biotransformation by chemical inhibitors**

Strong inhibition was observed with ketoconazole (CYP3A4 inhibitor, up to 80%). Other chemical inhibitors tested did not show significant inhibitory effects. The strong inhibition by ketoconazole and azamulin suggests the predominant role of CYP3A in the oxidative metabolism of capmatinib in HLM.

**TABLE 11 Chemical inhibition**

The IC50 values of CYP inhibitors on capmatinib metabolism in HLM were compared with the repotted Ki values with CYP-specific substrates

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Inhibitor (CYP)** | **Concentration range (μM)** | **Reported Ki or IC50 values(μM)** | | **IC50 (μM)** | **Maximal inhibition (%)** |
| **(a)** | **(b)** |
| **Furafylline (1A2)** | 0.16 – 10 | 2 (0.045-361.1) | 0.6-0.73 | >10 | 4 |
| **Montelukast (2C8)** | 0.008 – 2 | 0.014 (0.009-0.15) |  | >2 | 7 |
| **Sulfaphenazole (2C9)** | 0.08 – 50 | 0.51 (0.06-47) | 0.3 | >5 | 1 |
| **Ticlopidine (2C19)** | 0.16 – 10 | 1.7 (0.184-10) | 1.20 | >10 | 1 |
| **Quinidine (2D6)** | 0.008 – 2 | 0.0605 (0.00078-53) | 0.027-0.4 | >2 | 9 |
| **DETC (2E1)** | 1.56 – 100 | 13.7 (6-46) | 9.8-34 | >100 | 1 |
| **Ketoconazole (3A)** | 0.008 – 1 | 0.1 (0.001-32) | 0.004-0.18 | 0.10 | 80 |
| **Azamulin (3A)** | 0.04 – 5 | 0.15 (0.12-0.24) |  | 0.24 | 92 |
| **Methimazole (FMO)** | 2.5 – 160 |  | 61(c) | nd | nd |

nd, not determined

aNovartis data on file.

bValues from Food and Drug Administration (2006) guidance.

cKi value from Zhou et al, 2002.

**Fig. 7. Effect of CYP-selective inhibitors on capmatinib total metabolism in HLM**

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DETC, Sodium diethyldithiocarbamate; HLM, Human liver microsomes

**Correlation analysis in HLM from individual donors**

Correlation analysis by linear regression for [14C]capmatinib total metabolism and specific marker activities showed the best and the highest correlation for CYP3A4/5 with correlation coefficients (R) of 0.989 and 0.922 with testosterone 6β-hydroxylase and midazolam 1’-hydroxylase activities, respectively. All other cytochrome P450 and FMO-specific marker activities scored much lower correlation values, and thus, have no correlation with [14C]capmatinib metabolism in these microsomes.

**TABLE 12 Correlation analysis of capmatinib total metabolism rates with marker enzyme activities**

Correlation coefficient (R) of the rate of metabolism of 10 μM [14C]capmatinib (sum of all metabolites, pmol/min/mg, means of duplicates after 5 min incubation with 0.2 mg/mL HLM) with marker activities from 10 CYP enzymes and FMO in a set of 16 human liver microsomes from the Reaction Phenotyping Kit (Version 7, Cat No: H0500, XenoTech LLC, Kansas City, USA). Data are means of triplicates for enzymes as provided by the manufacturer (all activities in pmol/min/mg).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Human liver Nr.** | **Capmatinib metabolism rate** | **CYP 1A2 (a)** | **CYP**  **1A2 (b)** | **CYP 2A6 (c)** | **CYP 2B6 (d)** | **CYP 2B6 (e)** | **CYP 2C8 (f)** | **CYP 2C9 (g)** | **CYP 2C19 (h)** | **CYP 2D6 (i)** | **CYP 2E1 (j)** | **CYP 3A4/5 (k)** | **CYP 3A4/5 (l)** | **CYP 4A11 (m)** | **FMO**  **(n)** |
| 196 | 313 | 7.67 | 75.8 | 777 | 142 | 371 | 113 | 1020 | 48.5 | 468 | 1430 | 1930 | 562 | 1480 | 425 |
| 245 | 385 | 34.9 | 429 | 1350 | 129 | 433 | 249 | 1550 | 16.5 | 228 | 1960 | 2470 | 1050 | 2260 | 2000 |
| 258 | 184 | 21.5 | 234 | 804 | 45.3 | 46.1 | 11.4 | 1990 | 15 | 363 | 1250 | 1500 | 587 | 1030 | 1510 |
| 259 | 485 | 24.8 | 327 | 656 | 103 | 193 | 269 | 2480 | 44.9 | 117 | 3870 | 3180 | 1090 | 1310 | 833 |
| 273 | 80.0 | 43.8 | 486 | 1280 | 22.3 | 35 | 114 | 1330 | 21.6 | 128 | 1790 | 1240 | 442 | 1360 | 1220 |
| 276 | 266 | 31.8 | 316 | 646 | 47.2 | 153 | 186 | 1370 | 7.7 | 158 | 1890 | 2570 | 1200 | 1250 | 1820 |
| 277 | 266 | 97.3 | 643 | 1040 | 42.5 | 9.01 | 258 | 2490 | 169 | 191 | 2510 | 1510 | 478 | 2440 | 1830 |
| 287 | 333 | 42.9 | 514 | 1070 | 71.6 | 285 | 209 | 1370 | 3.39 | 240 | 3120 | 2050 | 781 | 1070 | 1170 |
| 288 | 1446 | 80.9 | 968 | 435 | 128 | 260 | 314 | 1380 | 46.6 | 495 | 2250 | 8000 | 3920 | 1350 | 2100 |
| 290 | 776 | 26.8 | 270 | 1630 | 192 | 838 | 170 | 662 | 748 | 121 | 1850 | 4100 | 1690 | 1170 | 814 |
| 292 | 1315 | 17.3 | 286 | 3130 | 247 | 546 | 445 | 1890 | 10.3 | 455 | 1980 | 7110 | 4040 | 1210 | 833 |
| 295 | 565 | 57.7 | 721 | 627 | 70.1 | 77.1 | 321 | 2810 | 42.4 | 133 | 4140 | 3140 | 2760 | 1910 | 1290 |
| 297 | 1414 | 9.42 | 114 | 566 | 77.9 | 24.1 | 210 | 2590 | 119 | 425 | 2180 | 8490 | 2910 | 2150 | 1030 |
| 298 | 166 | 14.7 | 162 | 945 | 45 | 112 | 132 | 1950 | 12.3 | 299 | 1410 | 788 | 242 | 1400 | 1600 |
| 300 | 1114 | 48.2 | 629 | 2960 | 193 | 420 | 376 | 3220 | 253 | 216 | 2270 | 6080 | 2380 | 1340 | 562 |
| 305 | 458 | 15.1 | 231 | 416 | 103 | 188 | 388 | 2040 | 6.61 | 301 | 2200 | 2490 | 1120 | 2000 | 1100 |
| pool | 623 | 34.4 | 401 | 1060 | 103 | 254 | 231 | 1690 | 104 | 251 | 2200 | 3540 | 1750 | 1480 | 1050 |
| **R** |  | **0.055** | **0.241** | **0.347** | **0.639** | **0.336** | **0.588** | **0.232** | **0.230** | **0.490** | **0.098** | **0.989** | **0.922** | **0.009** | **-0.183** |
| **R2** |  | **0.003** | **0.0579** | **0.121** | **0.408** | **0.113** | **0.346** | **0.054** | **0.0529** | **0.240** | **0.010** | **0.979** | **0.850** | **0.000** | **0.0335** |

**(a): 7-Ethoxyresorufin O-dealkylation, (b): Phenacetin O-deethylation, (c): Coumarin 7-hydroxylation, (d): S-Mephenytoin N-demethylation, (e): Bupropion hydroxylation, (f): Paclitaxel 6α-hydroxylation, (g): Diclofenac 4’-hydroxylation, (h): S-Mephenytoin 4’-hydroxylation, (i): Dextromethorphan O-demethylation, (j): Chlorzoxazone 6-hydroxylation, (k): Testosterone 6β- hydroxylation, (l): Midazolam 1’-hydroxylation, (m): Lauric acid 12-hydroxylation, (n): Benzydamine N-Oxidation**

Correlation analysis of specific marker activities with the individual metabolite formation rate of [14C]capmatinib was also carried out. Significant correlation was obtained with testosterone 6β-hydroxylation and midazolam 1’-hydroxylation for metabolites M8, M17, and M21 with correlation coefficients (R) of 0.984 and 0.921, 0.942 and 0.940, and 0.959 and 0.901, respectively (Supplementary Fig 8). The correlation results are in agreement with the inhibition data and confirmed that M8, M17, and M21 formation mainly resulted from CYP3A catalysis. The results of correlation analysis indicated that CYP3A is the major enzyme family involved in the hepatic microsomal metabolism of [14C]capmatinib.

**Fig. 8 Correlation of [14C]capmatinib metabolism rates with CYP3A4/5 marker enzyme activities (testosterone 6beta-hydroxylation)**

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## 5. Synthesis

**Preparation of reference compounds by biosynthesis**

The syntheses of the described oxidized phase I metabolites were carried out by incubating recombinant *E. coli* cells co-expressing human CYP 1A1 or 3A4 together with human reductase, bacterial strains, and liver homogenates from pig and foal.

**Preparative synthesis of metabolites M21 and M8**

From a frozen glycerol stock of recombinant *E. coli* expressing human CYP 3A4, CYP reductase, and cytochrome b5, some material was used to inoculate a preculture consisting of 8×200 mL MTB-2 medium in 500 mL shake flasks containing ampicillin and chloramphenicol at concentrations of 100 μg/mL and 50 μg/mL, respectively.

* + For MTB-2 medium:
* Bacto yeast extract (24 g/L) and bacto tryptone (14 g/L) were dissolved in distilled water and sterilized for 15 min at 121°C.
* K2HPO4, KH2PO4, and glycerol were dissolved in distilled water and the pH was adjusted to 6.8 and sterilized for 15 min at 121°C.
* Both media were combined, and sterile distilled water was added to attain the final concentrations. 1 mL/L of a 1 M thiamin and Trace element 4000× solution were added under sterile conditions.
  + For Trace element stock solution 4000×
* Fe (III) citrate (24.5 g/L) was dissolved under heating, followed by addition of concentrated HCl (0.1 L/L), ZnCl2 (1.31 g/L), CoCl2.6H2O (2 g/L), Na2MoO4.2H2O (2 g/L), CaCl2.2H2O (1 g/L), CuCl2.2H2O (1.27 g/L), and H3BO3 (0.5 g/L) and dissolved. The solution was sterilized by filtration.
  + For 1 M thiamin and trace element 4000X solution
* The solution of thiamin hydrochloride (337 g/L) and trace element stock solution 4000X (250 mL/L) were sterilized by filtration and stored at −20 °C.

The preculture was incubated overnight at 30°C and 180 rpm. A volume of 1600 mL pre-culture was transferred into a 50 L fermenter filled with 35 L MTB-2 medium. Cells were incubated at 30°C, with stirring at 80 rpm and aeration with pressurized air at 0.5 volume per volume and minute (vvm). pH was adjusted to 6.8 with 12.5% NH4OH. After 8 h of incubation, the culture was fed with a solution consisting of 248 g/L glycerol and 171 g/L yeast extract with a feed rate of 2 mL/min. After 20 h of incubation, the culture reached an optical density at 600 nm (OD600) of 2.1. At this time point, the culture was induced by adding isopropyl-β-D-thiogalactopyranosid (IPTG) and *δ* -Aminolevulinic acid (d-Ala) to a final concentration of 1 mM and 0.5 mM, respectively. After 58 h of cultivation, the cells were centrifuged and washed three times with PSE buffer. In order to remove the remaining indol, which was formed during the cultivation, approximately 150 g of Amberlite® XAD16N adsorbent resin (XAD) were added and the culture stirred for 1 hour. XAD was removed by filtration and the cell culture was diluted with PSE buffer to a final OD of OD600 = 100. The cells were stored at −80 ⁰C until usage for preparative scale biotransformation.

In order to start the metabolite synthesis, 500 mL of *E. coli* cell suspension expressing rh CYP 3A4 together with human CYP reductase and human cytochrome b5 were thawed and mixed with 500 mL PSE buffer, 20 mL of a 50% (w/v) glycerol solution, and 10 mL of a 10 mg/mL capmatinib solution in 1:9 dimethyl sulfoxide (DMSO)/Methanol. The final concentration of capmatinib and glycerol was 0.1 mg/mL and 1%, respectively. The suspension was mixed using a paddle agitator, and 250 mL of the biotransformation mixture was transferred into 2 L Erlenmeyer shake flasks with four baffles (four flasks in total). The flasks were sealed using breathable seals and incubated at 30 ⁰C and 155 rpm. The metabolite formation was assessed using HPLC. Two different biotransformation products have been produced during this biotransformation.

After 3 h of incubation, no further conversion of capmatinib into the corresponding biotransformation products was observed. The reaction was stopped by adding 12.5 g XAD into each flask and further incubated for 1 hour at 60 rpm and 20°C. Further, the broth was filtered with gauze. The XAD particles were transferred into a glass column closed with cotton wool and the biotransformation products as well as remaining capmatinib were eluted with methanol. The eluate was concentrated under reduced pressure. The concentrate was mixed with 15 g of diatom granulates (Isolute HM-N, Biotage) in order to absorb the biotransformation substrate and product molecules and was further evaporated to dryness.

The biotransformation products were purified by reversed-phase HPLC (RP-HPLC). Therefore, the dry isolute was stacked above a reverse C18 column. The column was eluted with a 1‰ formic acid/acetonitrile (ACN) step gradient. Product was eluted with 15% ACN. The product containing fractions were concentrated under reduced pressure giving the raw product. The main fractions containing the two biotransformation products M21 and M8 were treated separately from here on.

For the second chromatographic run, both the raw products were dissolved in DMSO. Charges of 2 mL were injected on a C18 reverse phase column and eluted using a 5−40% ACN gradient in 4 mM ammonium formate (pH 5.5). The products eluted at around 20−30% ACN. The fractions were analyzed by HPLC, and all fractions containing the product were combined and concentrated under reduced pressure. Residual water was removed by lyophilization overnight. The purity of biotransformation products was determined by HPLC-UV (215 nm).

**Preparative synthesis of metabolites M17 and M18**

From a frozen glycerol stock of recombinant *E. coli* expressing human CYP 1A1 and CYP reductase, some material was used to inoculate a preculture consisting of 5×200 mL LB medium in 500 mL shake flasks containing ampicillin at a concentration of 100 μg/mL. The pre-culture was incubated overnight at 37°C and 180 rpm. LB medium was prepared by dissolving peptone from casein (10 g/L), yeast extract (5 g/L), and NaCl 10 g/L in distilled water and sterilizing at 121°C for 15 min in an autoclave. A pre-culture (1 L) was transferred into a 50 L fermenter filled with 20 L Riesenberg medium. The Riesenberg medium is composed of (NH4)2SO4 (2.5 g/L), Fe (III) citrate (0.03 g/L), MgCl2.7H2O (1 g/L), CaCl2 (0.03 g/L), and trace elements solution (100×) (10 mL/L). Trace elements solution is composed of CoCl2.6H2O (0.267 g/L), CuCl2.2H2O (0.151 g/L), MnCl2 (0.102 g/L), H3BO3 (0.333 g/L), Na2MoO4.2H2O (0.267 g/L), and ZnSO4.7 H2O (1.09 g/L). The solution was sterilized by filtration.

Cells were incubated at 30°C, stirred at 300 rpm, and aerated with pressurized air at 1 vvm. pH was adjusted to 6.8 with NH4OH 12.5%. After 6 h of incubation, the culture reached an optical density of OD600 = 2. At this time point, the culture was induced by adding IPTG and d-Ala to a final concentration of 1 mM and 0.5 mM, respectively. Immediately after induction, the temperature was set to 25°C. The stirrer speed was increased gradually until 500 rpm, but without exceeding dissolved oxygen >0%, so that the growth was limited by oxygen. After 24 h of incubation, the culture was fed with a 25% (w/w) glycerol solution at a feed rate of 0.75 mL/min. After 48 h of cultivation, the cells were centrifuged and washed three times with PSE buffer and extracted with XAD to remove indol, as described previously. The cell culture was diluted with PSE buffer to a final OD of OD600 = 100. The cells were stored at −80°C until usage for preparative scale biotransformation. In order to start the metabolite synthesis, 1 L of *E. coli* cell suspension expressing rh CYP 1A1 together with human CYP reductase was thawed and mixed with 1 L PSE buffer, 40 mL of a 50% (w/v) glycerol solution, and 20 mL of a 10 mg/mL capmatinib solution in 1:9 DMSO/methanol. The final concentration of capmatinib and glycerol were 0.1 mg/mL and 1%, respectively. The suspension was mixed using a paddle agitator. Subsequently, 250 mL of the biotransformation mixture were transferred into 2 L Erlenmeyer shake flasks with four baffles (eight flasks in total). The flasks were sealed using breathable seals and incubated at 30°C and 210 rpm. The reaction was monitored using HPLC.

If the yield was not satisfying after 6 h, 20 mL nutrient solution (composed of trisodium citrate [60 g/L], glucose [40 g/L], and lactate monohydrate [40 g/L] dissolved in 1L of PSE buffer] was added to four of the flasks. This proved to be beneficial for the production of M17, and 20 mL nutrient solution was also added to the other flasks. After 18.5 h, the reaction was stopped by freezing the culture at −25°C. Three different biotransformation products have been produced during this process. The downstream processing and purification of biotransformation products have been performed as described before for the metabolite synthesis with CYP 3A4. The purity of biotransformation products was determined by HPLC-UV (215 nm).

**Preparative synthesis of metabolite M3 by microbial biotransformation**

A pre-culture of the microbial strain ATCC55043 (*Streptomyces sp*.) was grown in 3 × 20 mL NL148sb medium in 100 mL shake flasks at 28°C and 220 rpm for 2 days. For NL148sb medium, soluble starch (20 g/L), casein digest (4 g/L), Bacto Soytone (4 g/L), LabLemco Powder (4 g/L), Bacto Yeast extract (0.5 g/L), NaCl (1.5 g/L), and MOPS (21 g/L) were dissolved in distilled water, and the pH was adjusted to 7.5. After sterilization in an autoclave for 20 min at 121°C, 1 mL/L trace element solution A was added.

Trace element solution A was composed of H3BO3 (0.1 g/L), FeSO4.7H2O (5 g/L), potassium iodide (0.05 g/L), CoCl2.6H2O (0.2 g/L), CuSO4.5H2O (0.2 g/L), MnCl2.4H2O (2 g/L), ZnSo4.7H2O (4 g/L), and H2SO4 96%(1 mL/L). The solution was sterilized by filtration. Sterile glycerol was added to each pre-culture to a final concentration of 16.7% (w/v). The pre-cultures were stored at −80°C to inoculate the main cultures later. 12.5 mL of thawed pre-culture was then used to inoculate 250 mL medium in a 1-L shake flask. Four flasks were cultivated in parallel to give a final biotransformation volume of 1 L. The cultures were incubated at 28°C and 200 rpm. After 2 days of incubation, capmatinib was added to each flasks to a final concentration of 0.1 mg/mL. The biotransformation processes were monitored using HPLC.

After 2 days, the biotransformation mixture was centrifuged at 12000*g* for 15 min. The cell pellet was extracted three times: once with 500 mL MeOH/ACN 1:1 and two times with 500 mL MeOH. The extract was evaporated under reduced pressure. The supernatant of the biotransformation mixture was stirred at 150 rpm with 50 mg XAD resin for 1 h. XAD was filtered over cotton, and the filtrate was discarded. The biotransformation substrate and product were eluted from XAD by washing XAD with MeOH/ACN 1:1 until no substrate or product was detected by HPLC anymore. The eluate was combined with the concentrated extract from cell pellet and further concentrated under reduced pressure. The concentrate was mixed with 15 g of diatom granulates, and all solvent was evaporated under reduced pressure. The biotransformation product was purified via two RP-HPLC runs as described before for the biotransformation process with rh CYP 3A4. The purity of the biotransformation product was determined by HPLC-UV (215 nm).

**Preparative synthesis of Metabolite M16 by microbial biotransformation**

A pre-culture of the microbial strain ATCC31561 (*Streptomyces diastatochromogenes*) was grown in 100 mL NL148sb medium in a 500 mL shake flask at 28°C and 220 rpm for 3 days. Sterile glycerol was added to the pre-culture to a final concentration of 16.7% (w/v). The preculture was stored at −80°C to inoculate the main cultures later. 2 mL of thawed pre-culture was then used to inoculate 500 mL medium in a 2-L shake flask. Two flasks were maintained in parallel to give a final biotransformation volume of 1 L. Capmatinib was added to a final concentration of 0.1 mg/mL right at the beginning of the incubation. Cell cultures were incubated at 28°C and 220 rpm. The biotransformation process was monitored using HPLC. After three days, the biotransformation mixture was stopped. The following purification of the biotransformation product was performed in the same ways as described for the product of the microbial biotransformation with ATCC 55043. The purity of the biotransformation product was determined by HPLC -UV (215 nm).

**Preparation of reference compounds (M13 and M19) by chemical synthesis**

**Step-1**

A solution of 2-fluoro-4-(7-(quinolin-6-ylmethyl)imidazo[1,2-b][1,2,4]triazin-2-yl)benzonitrile (**1**) (10 g, 26.29 mmol) in concentrated HCl (100 mL) and water (20 mL) was heated to 105°C and stirred for 16 h.

The reaction mixture was then concentrated under reduced pressure, and the residue was diluted with water. The solid obtained was filtered, washed with water, and dried to obtain compound **2 (Metabolite M13, Fig 9)** as a yellow solid (9.2 g, 87.6%).

**Step-2**

To a mixture of compound **2** (8 g, 20.03 mmol) and PyBOP (12.5 g, 24.02 mmol) in dimethyl formamide (DMF) (100 mL) at 0°C, methylamine (2 M solution in tetrahydrofuran [THF], 40 mL, 80 mmol) was added followed by triethylamine (8.4 mL, 60.27 mmol) and the reaction mixture was stirred at room temperature for 3 h.

The reaction mixture was then concentrated under reduced pressure, and the residue was added with water. The solid obtained was filtered, washed with water, and dried to get compound **3** as a yellow solid (13.1 g, 79.3%).

**Step-3**

To a stirred solution of compound **3** (13 g, 31.52 mmol) in dichloromethane (500 mL) and methanol (100 mL) at 0°C, 3-chloroperbenzoic acid (~75%; 11 g, 47.81 mmol) was added and the reaction mixture was stirred at room temperature for 16 h.

The reaction mixture was quenched with saturated sodium sulfite solution and saturated sodium bicarbonate solution at 0°C. The precipitated solid was filtered, washed with water and then with acetonitrile, and dried to get compound **4** as a yellow solid (11.5 g, 85.1%).

**Step-4**

To a suspension of compound **4** (1.9 g, 4.435 mmol) in dichloromethane (50 mL) at 0°C, potassium carbonate (10% aqueous solution, 20 mL, 14.47 mmol) was added followed by *p*-toluenesulfonyl chloride (1 g, 5.245 mmol) and the reaction mixture was stirred at room temperature for 3 days.

The reaction mixture was concentrated under reduced pressure and diluted with water. The solid obtained was filtered, washed with water and then with acetonitrile, and dried to obtain compound **5** (Fig 9) as a yellow solid. (9.5 g; ~75% pure by LC-MS).

**Note**: The crude product thus obtained was mixed with the crude product obtained in other batches and purified by preparative RP-HPLC (0.1% trifluoracetate [TFA] + CH3CN), and the fractions were concentrated under reduced pressure and dried to get **Metabolite M19** (TFA salt) as a yellow solid.

**Fig. 9. Schematic presentation of the synthesis of the M13 (2) and M19 (5)**

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MS and NMR data of the authentic reference compounds: Metabolite

M3 (A), Metabolite M8 (B), Metabolite M13 (C), Metabolite M16 (D), Metabolite M17 (E),

Metabolite M18 (F), Metabolite M19 (G), Metabolite M21

## 6. NMR spectra

**A. Metabolite M3**

**Molecular Formula: C23H17FN6O3**

**Average Mass: 444.42**

**Monoisotopic Mass: 444.13462**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O3 445.1419; Found 445.1420.

1H NMR (600 MHz, DMSO-d6) δ 12.46 (br., 1H), 8.89 (dd, J = 4.5, 1.7 Hz, 1H), 8.41 (dd, J = 8.3, 1.7 Hz, 1H), 8.34 (q, J = 5.2 Hz, 1H), 8.15 (d, J = 2.0 Hz, 1H), 8.01 (d, J = 8.7 Hz, 1H), 7.95 (d, J = 12.6 Hz, 1H), 7.93 (d, J = 8.6 Hz, 1H), 7.84 (dd, J = 8.8, 2.0 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.55 (dd, J = 8.3, 4.2 Hz, 1H), 7.14 (s, 1H), 6.45 (s, 1H), 6.25 (s, 1H), 2.79 (d, J = 4.6 Hz, 3H).

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**B. Metabolite M8**

**Molecular Formula: C23H17FN6O2**

**Average Mass: 428.42**

**Monoisotopic Mass: 428.13970**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O2 429.1470; Found 429.1479.

1H NMR (600 MHz, DMSO-d6) δ 9.24 (s, 1H), 8.88 (dd, J = 4.4, 1.7 Hz, 1H), 8.44 – 8.37 (m, 2H), 8.21 (d, J = 2.1 Hz, 1H), 8.05 – 8.01 (m, 2H), 8.00 (d, J = 8.7 Hz, 1H), 7.90 (s, 1H), 7.88 (dd, J = 8.7, 2.0 Hz, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.54 (dd, J = 8.2, 4.2 Hz, 1H), 6.58 (s, br, 1H), 6.54 (s, 1H), 2.81 (d, J = 4.6 Hz, 3H).

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**C. Metabolite M13**

**Molecular Formula: C22H14FN5O2  
Average Mass: 399.38  
Monoisotopic Mass: 399.11315**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C22H15FN5O2 400.1204; Found 400.1210.

1H NMR (600 MHz, DMSO-d6) δ 9.28 (s, 1H), 9.16 (d, J = 5.0 Hz, 1H), 8.93 (d, J = 8.4 Hz, 1H), 8.27 (d, J = 8.8 Hz, 1H), 8.24 (s, 1H), 8.11 (d, J = 8.8 Hz, 1H), 8.08-8.01 (m, 4H), 7.93 (dd, J = 8.4, 5.1 Hz, 1H), 4.73 (s, 2H).

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HCO2H

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**D. Metabolite M16**

**Molecular Formula: C23H17FN6O2**

**Average Mass: 428.42**

**Monoisotopic Mass: 428.13970**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O2 429.1470; Found 429.1464.

1H NMR (600 MHz, DMSO-d6) δ 12.50 (s, 1H), 8.85 (d, J = 4.1 Hz, 1H), 8.35 – 8.26 (m, 2H), 8.00 – 7.92 (m, 3H), 7.93 – 7.90 (m, 1H), 7.76 (dd, J = 8.7, 2.1 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.51 (dd, J = 8.3, 4.1 Hz, 1H), 7.25 (s, 1H), 4.36 (s, 2H), 2.78 (d, J = 4.4 Hz, 3H).

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**E. Metabolite M17**

**Molecular Formula: C23H17FN6O2**

**Average Mass: 428.42**

**Monoisotopic Mass: 428.13970**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O2 429.1470; Found 429.1464.

1H NMR (600 MHz, DMSO-d6) δ 9.22 (s, 1H), 9.03 (t, J = 4.4 Hz, 1H), 8.85 (dd, J = 4.3, 1.7 Hz, 1H), 8.31 (dd, J = 8.4, 1.8 Hz, 1H), 8.14 (s, 1H), 8.08 – 8.03 (m, 2H), 8.00 (s, 1H), 7.97 (d, J = 8.8 Hz, 1H), 7.84 – 7.76 (m, 2H), 7.50 (dd, J = 8.3, 4.1 Hz, 1H), 5.80 (t, J = 6.7 Hz, 1H), 4.71 (t, J = 6.5 Hz, 2H), 4.65 (s, 2H).

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**F. Metabolite M18**

**Molecular Formula: C22H15FN6O**

**Average Mass: 398.39**

**Monoisotopic Mass: 398.12914**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C22H16FN6O 399.1364; Found 399.1375.

1H NMR (600 MHz, DMSO-d6) δ 9.26 (s, 1H), 9.10 (dd, J = 4.8, 1.6 Hz, 1H), 8.79 (d, J = 8.4 Hz, 1H), 8.18 (d, J = 2.0 Hz, 1H), 8.14 (d, J = 8.8 Hz, 1H), 8.06 – 8.00 (m, 4H), 7.87 – 7.84 (m, 2H), 7.83 (t, J = 6.3 Hz, 1H), 7.78 (s, 1H), 4.71 (s, 2H).

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**G. Metabolite M19**

**Molecular Formula: C23H17FN6O2**

**Average Mass: 428.42**

**Monoisotopic Mass: 428.13970**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O2 429.1470; Found 429.1484.

1H NMR (400 MHz, DMSO-d6) δ 11.72 (s, 1H), 9.25 (s, 1H), 8.52 – 8.35 (m,br 1H), 8.06 (d, J = 9.7 Hz, 2H), 7.96 (s, 1H), 7.84 (d, J = 9.6 Hz, 1H), 7.82 (t, J = 7.8 Hz, 1H), 7.66 (d, J = 1.9 Hz, 1H), 7.53 (dd, J = 8.3, 2.0 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 6.47 (d, J = 9.6 Hz, 1H), 4.46 (s, 2H), 2.81 (d, J = 4.6 Hz, 3H).





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**Metabolite M21**

**Molecular Formula: C23H17FN6O2**

**Average Mass: 428.42**

**Monoisotopic Mass: 428.13970**

HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for C23H18FN6O2 429.1470; Found 429.1471.

1H NMR (600 MHz, DMSO-d6) δ 9.23 (s, 1H), 8.52 (dd, J = 6.0, 1.0 Hz, 1H), 8.47 (d, J = 8.9 Hz, 1H), 8.40 (q, J = 3.7 Hz, 1H), 8.06 (s, 1H), 8.04 – 8.02 (m, 2H), 8.01 (s, 1H), 7.87 (d, J = 8.5 Hz, 1H), 7.83 (dd, J = 9.0, 1.9 Hz, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.44 (dd, J = 8.5, 6.0 Hz, 1H), 4.66 (s, 2H), 2.80 (d, J = 4.6 Hz, 3H).

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accessible at <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/clinical-drug-interaction-studies-study-design-data-analysis-and-clinical-implications-guidance>

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