Reactive intermediates formation and bioactivation pathways of acalabrutinib revealed by LC-MS/MS: *in vitro* metabolic study

Supplementary data

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1- Chromatographic condition

Table 1: Summary of optimized LC-ITMS parameters.

<table>
<tr><td rowspan="3">Binary mobile phase</td><td colspan="2">0.1 % Formic acid in H_2O (A) and ACN (B)</td><td rowspan="3">ESI source</td><td>Positive ESI</td></tr>
<tr><td colspan="2">0.5 mL/minute</td><td rowspan="2">Drying gas (N_2 gas) at pressure (60 psi) and flow rate of 10 L/minute</td></tr>
<tr><td colspan="2">Elution time: 45 minutes</td></tr>
<tr><td rowspan="3">Agilent Zorbax eclipse plus C_{18} Column</td><td>Length</td><td>150 mm</td><td rowspan="2"></td><td>ESI T: 350 ºC</td></tr>
<tr><td>ID</td><td>4.6 mm</td><td>Capillary voltage: 4000 V</td></tr>
<tr><td>Particle size</td><td>3.5 µm</td><td>Modes</td><td>Mass scan and MS^2</td></tr>
<tr><td rowspan="2"></td><td>T</td><td>22±1ºC</td><td>Collision gas</td><td>High purity N_2</td></tr>
<tr><td rowspan="7">Gradient elution system</td><td>Time in minutes</td><td>% ACN</td><td>Analytes</td><td>ACB and its in vitro metabolites and RIs.</td></tr>
<tr><td>0</td><td>5</td><td rowspan="6">Mass features</td><td rowspan="6">Fragmentor voltage (FV): 145 V
Amplitude: 1.25 V</td></tr>
<tr><td>15</td><td>30</td></tr>
<tr><td>20</td><td>40</td></tr>
<tr><td>25</td><td>50</td></tr>
<tr><td>30</td><td>50</td></tr>
<tr><td>45</td><td>5</td></tr>
</table>

2- Identification of M2.

M2 (*m/z* 480) is proposed to be generated by oxidation of ACB. This metabolite was predicted by StarDrop WhichP450 module. This metabolite's peak elutes at 19.1 minute in fragment ion chromatogram. Dissociation of M2 ion at inside the collision cell generates six fragment ions at *m/z* 462, *m/z* 444, *m/z 414,* m/*z* 395, m/*z* 385 and *m/z* 367 (Figure S1). Predicted toxicities of M2 by DEREK Nexus module showed that oxidation did not change the toxicity alert for skin sensitization.

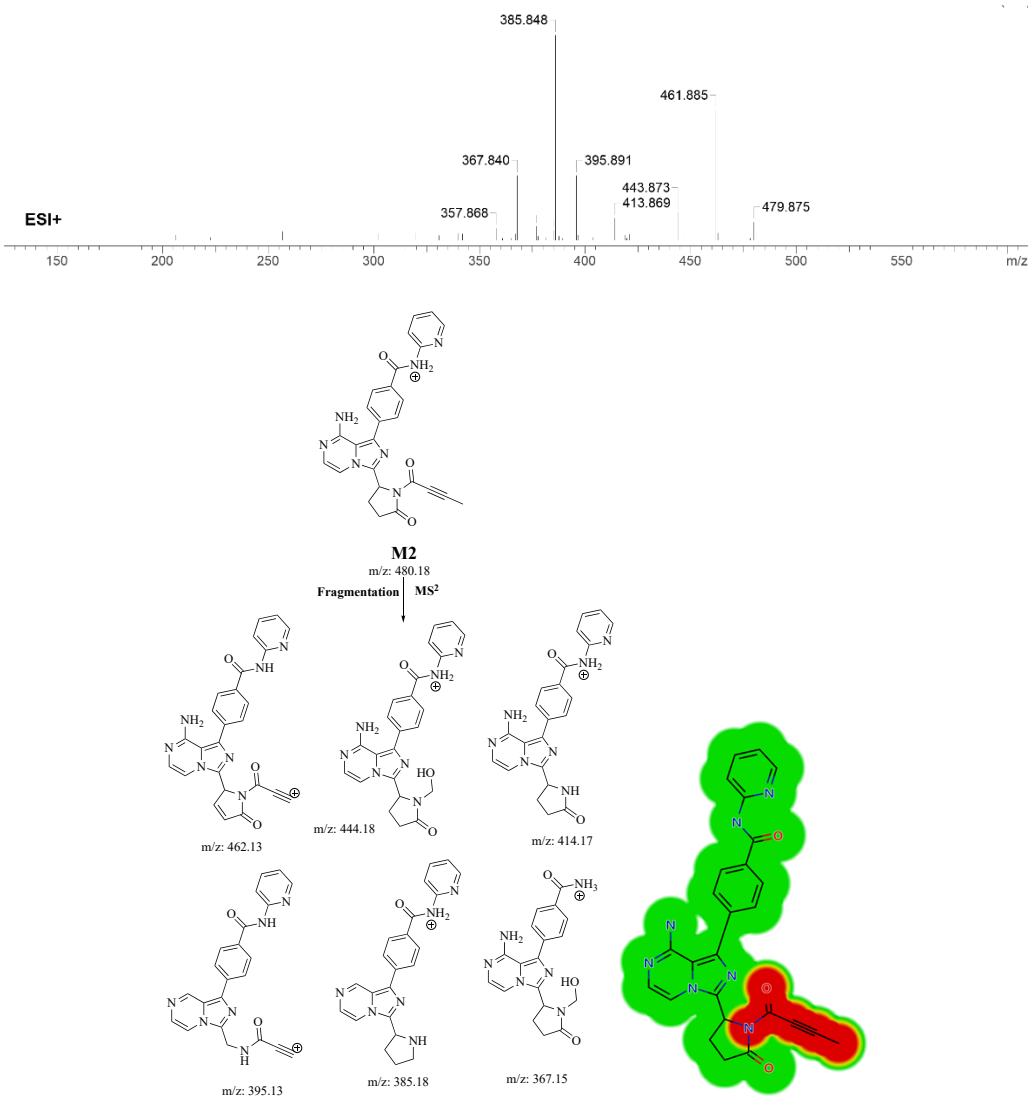

Figure S1. Product ion mass spectrum of M2, the proposed interpretation of fragmentation of M2 and the precited toxicities by StarDrop DEREK Nexus module.

3- Identification of M3.

M3 (*m/z* 400) is proposed to be generated by N-dealkylation of ACB. This metabolite's peak elutes at 18.46 minute in fragment ion chromatogram. Dissociation of M3 ion inside the collision cell generates five fragment ions at *m/z* 386, *m/z* 383, *m/z 331,* *m/z* 306 and *m/z* 237 (Figure S2). DEREK module predicted that n-dealkylation of ACB remove the alert of skin sensitization but add the risk of HERG channel inhibition (plausible).

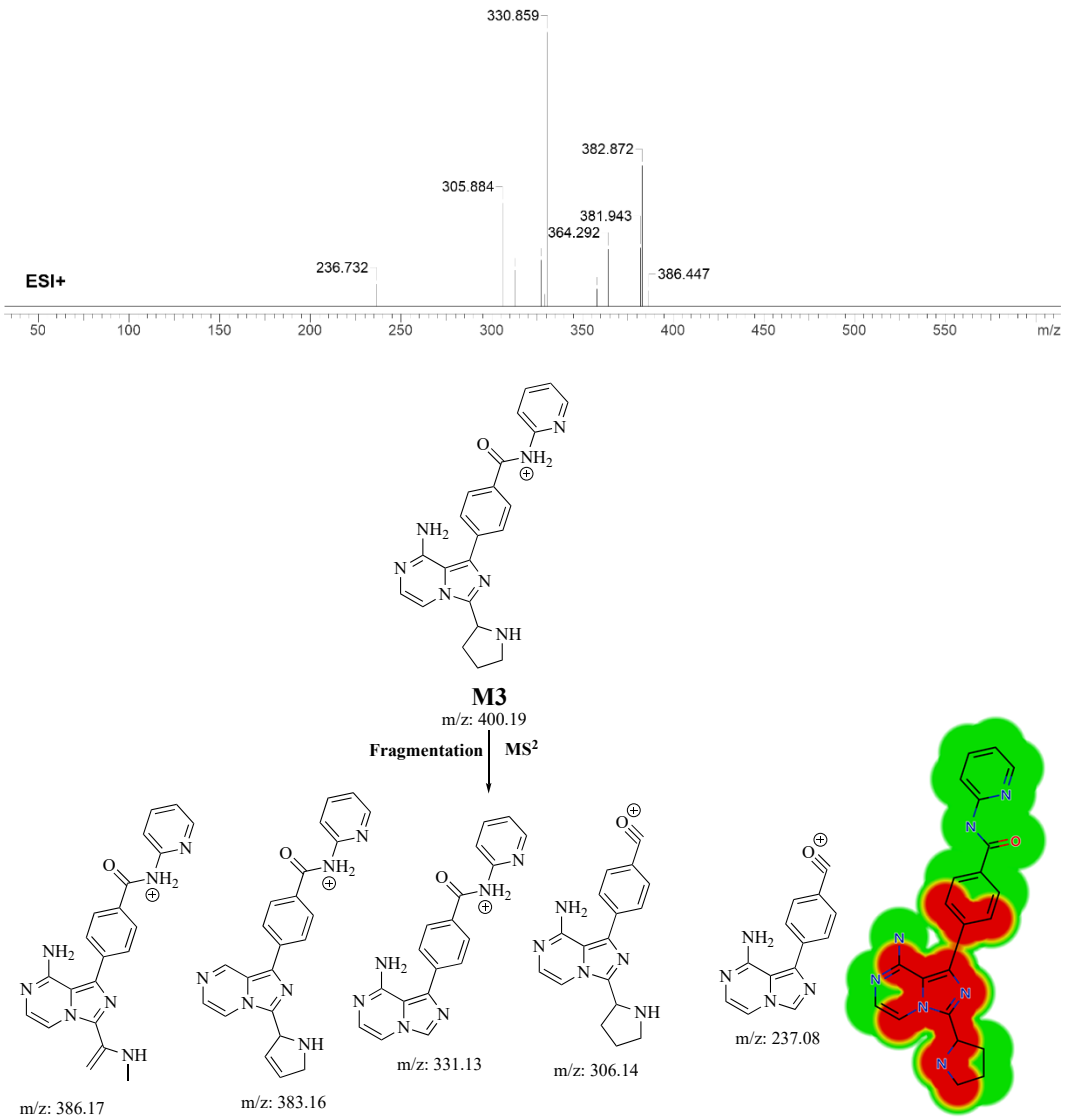

Figure S2. Product ion mass spectrum of M3 and the proposed interpretation of fragmentation of M3.

4- Identification of M4.

M4 (m/z 416) is proposed to be generated by hydroxylation and n-dealkylation of ACB. This metabolite's peak elutes at 23.05 minute in fragment ion chromatogram. Dissociation of M4 ion inside the collision cell generates four fragment ions at *m/z* 398, *m/z* 370, *m/z 331* and *m/z* 322 (Figure S3).

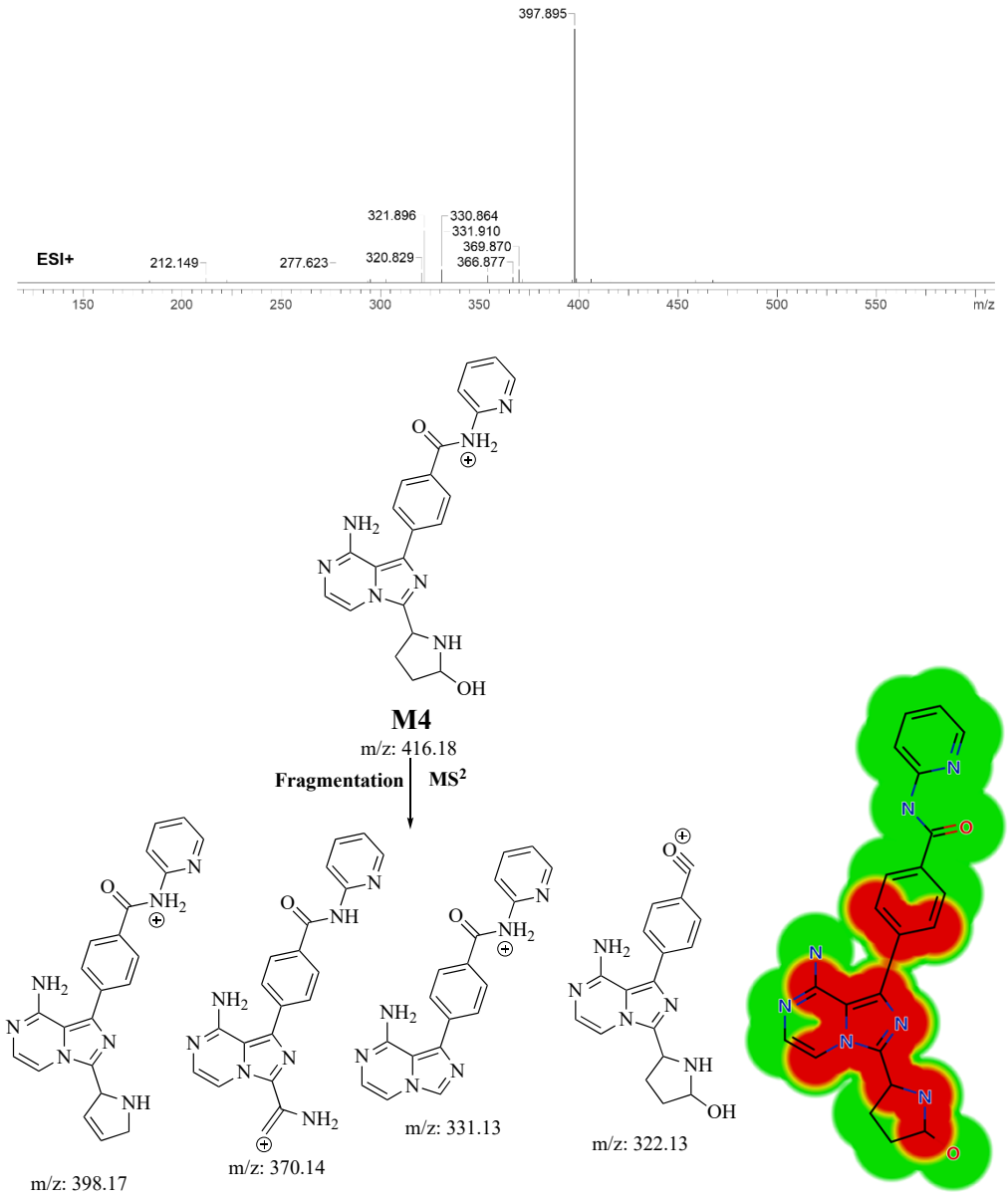

Figure S3. Product ion mass spectrum of M4, the proposed interpretation of fragmentation of M4.

5- Identification of M5.

M5 (m/z 444) is proposed to be generated by of ACB. This metabolite's peak elutes at 19.79 minute in fragment ion chromatogram. Dissociation of M5 ion inside the collision cell generates five fragment ions at *m/z* 426, *m/z* 406 *m/z* 331, *m/z* 382 and *m/z* 369 (Figure S4).

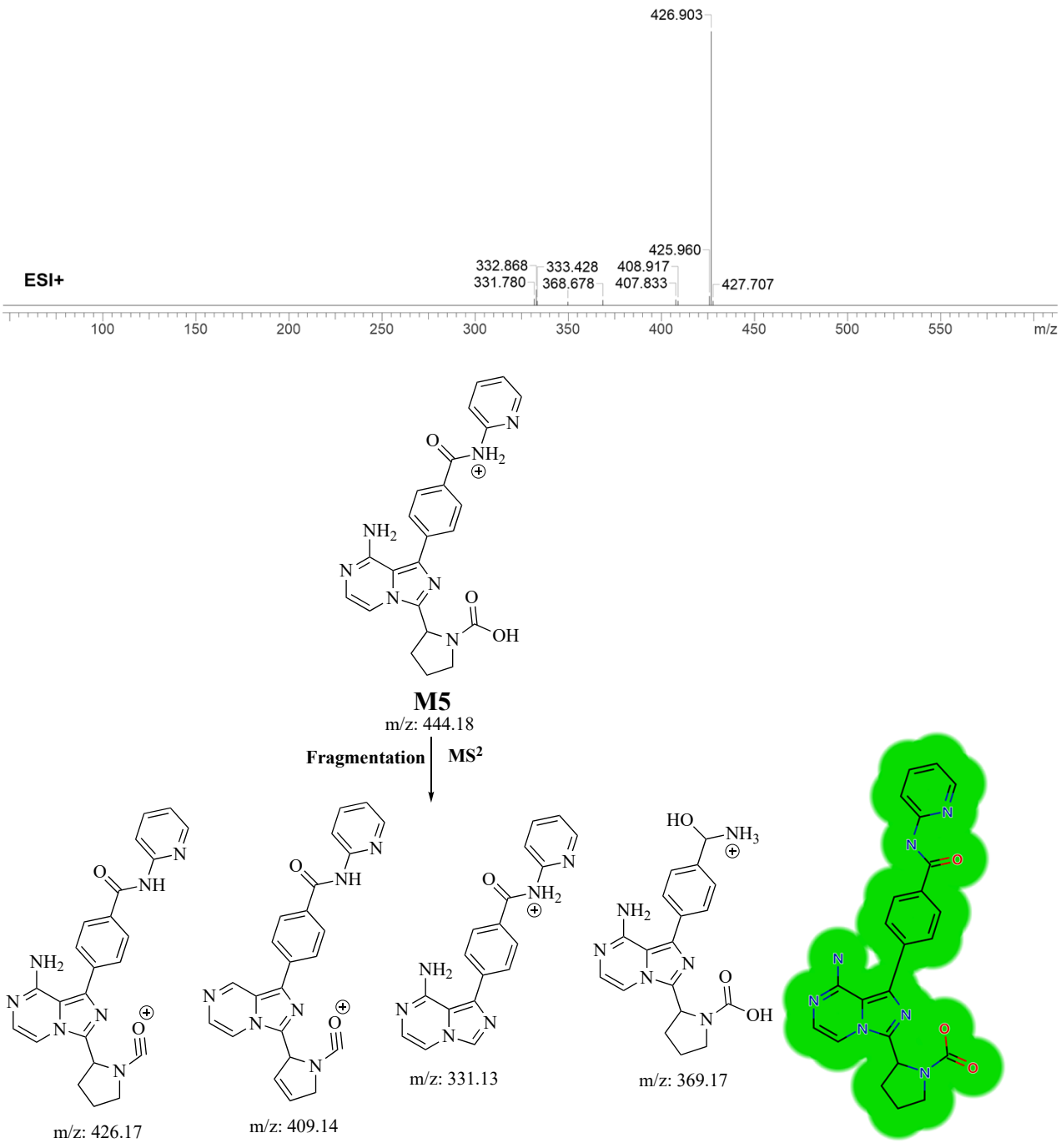

Figure S4. Product ion mass spectrum of M5 and the proposed interpretation of fragmentation of M5.