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Protocol extension

A versatile toolkit for drug metabolism studies with GNPS2: from drug development to clinical monitoring

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A Versatile Toolkit for Drug Metabolism Studies with GNPS2: from Drug Development to Clinical Monitoring

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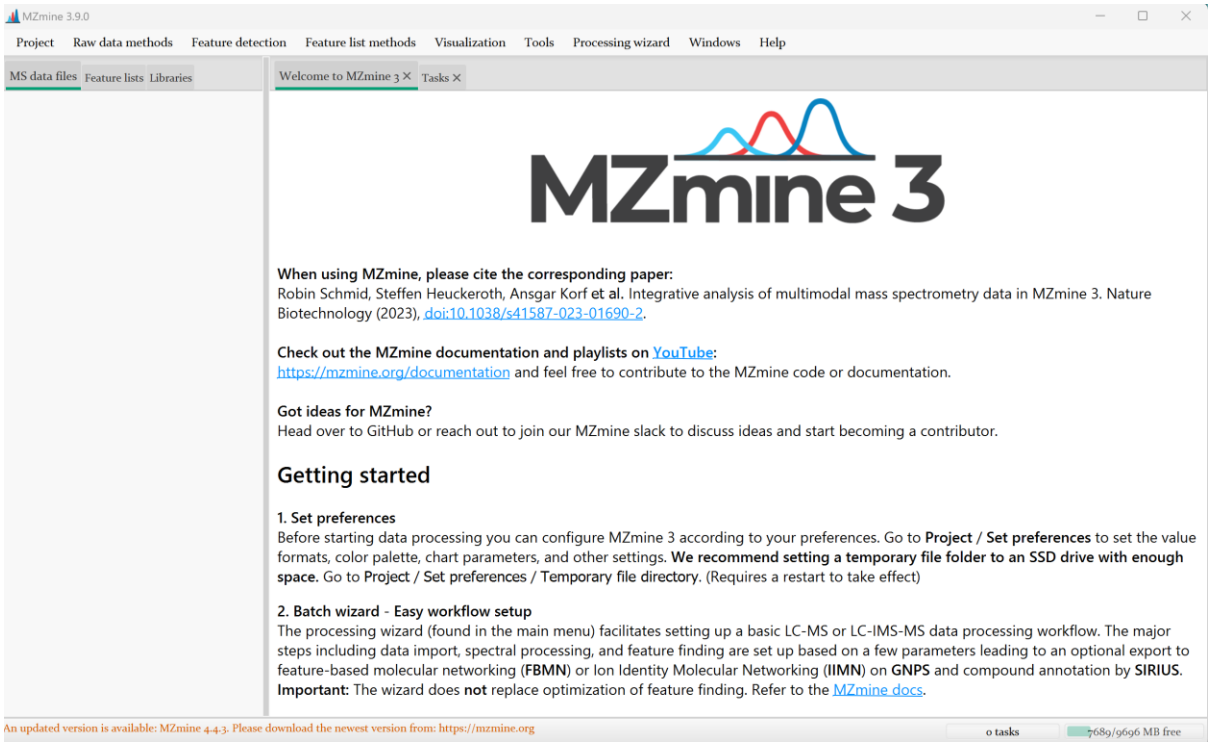
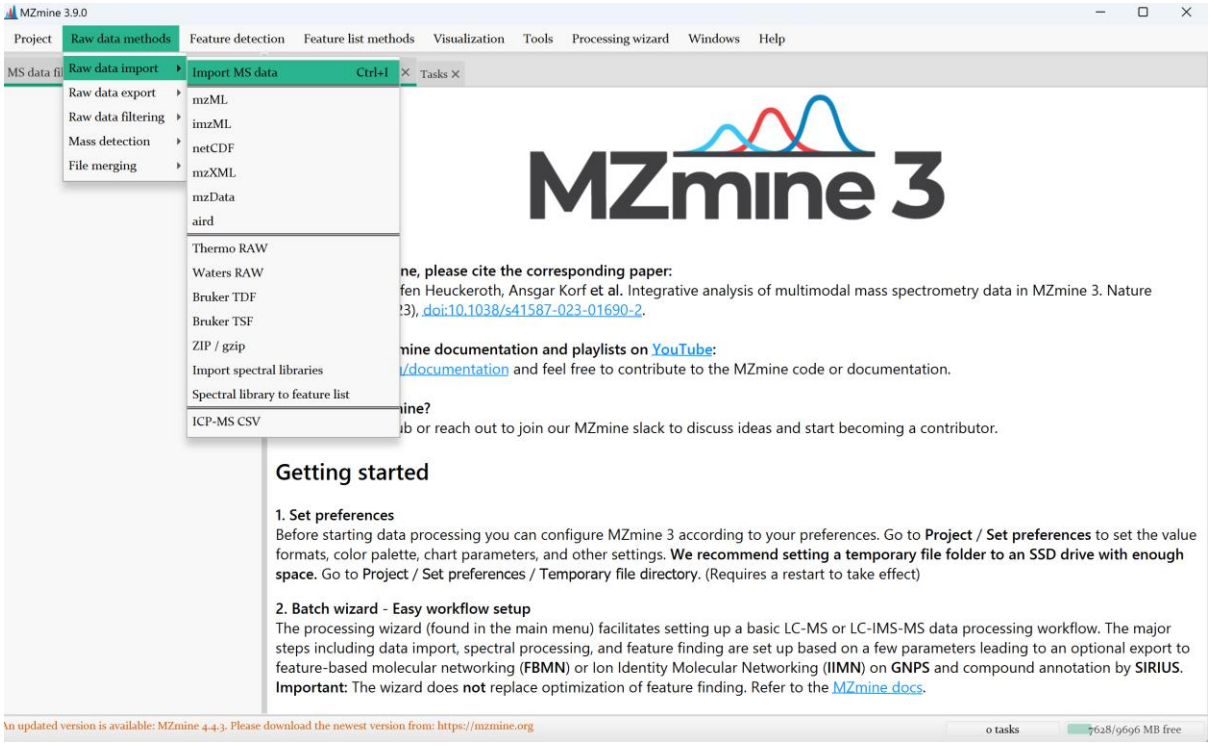
Supplementary Figures

Supplementary Figure 1. Step-by-step screenshots of feature detection through mzmine

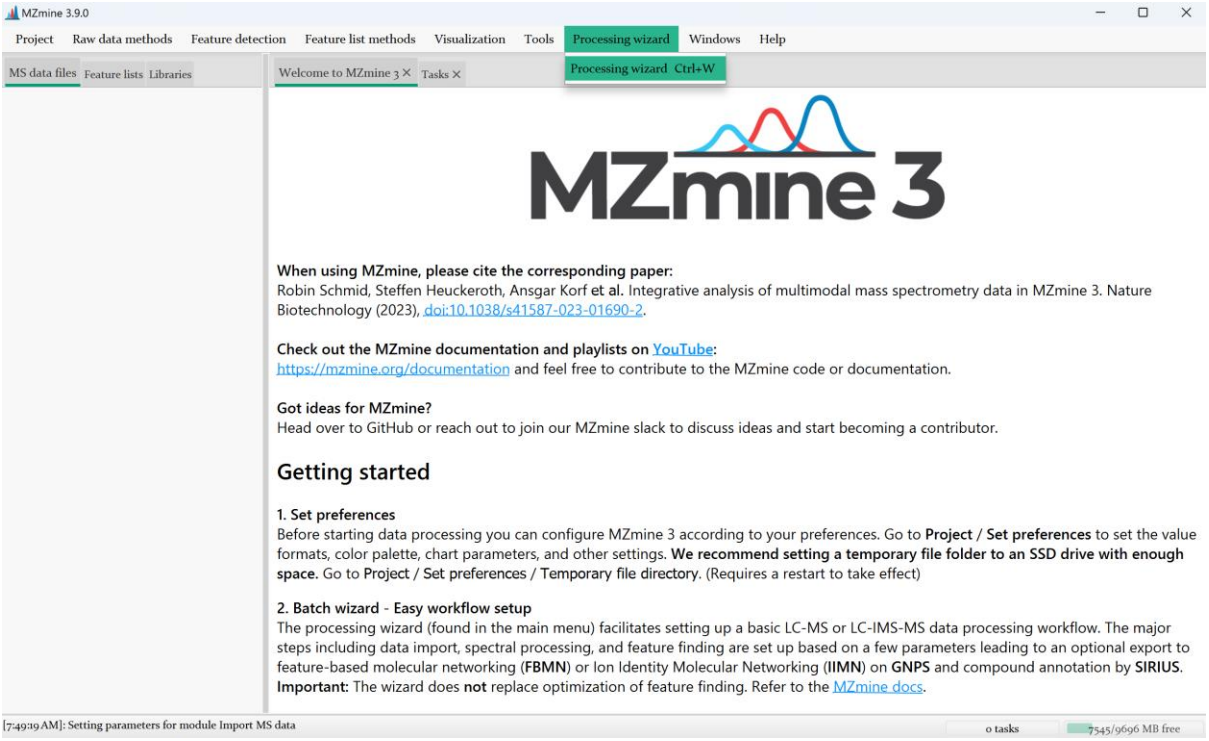
(a) Run mzmine.



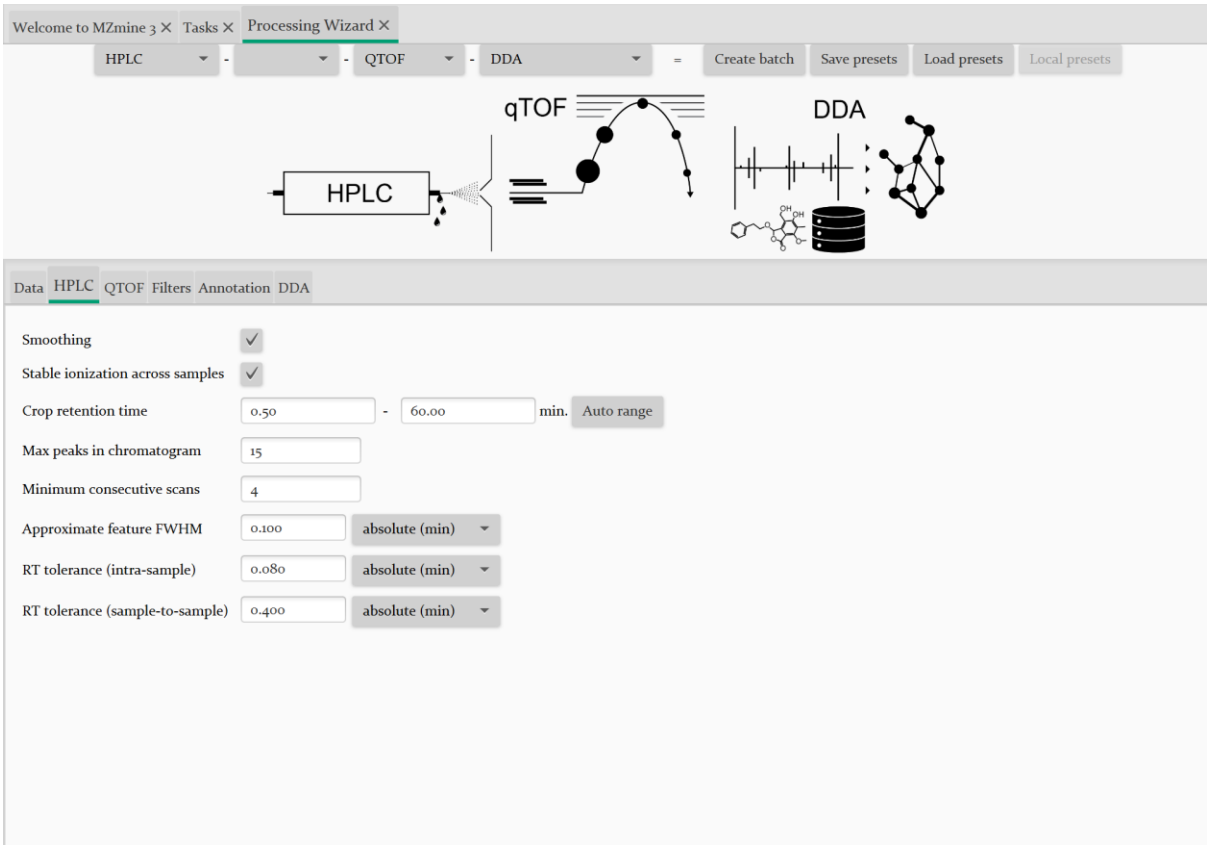
(b) Import converted data (mzML).



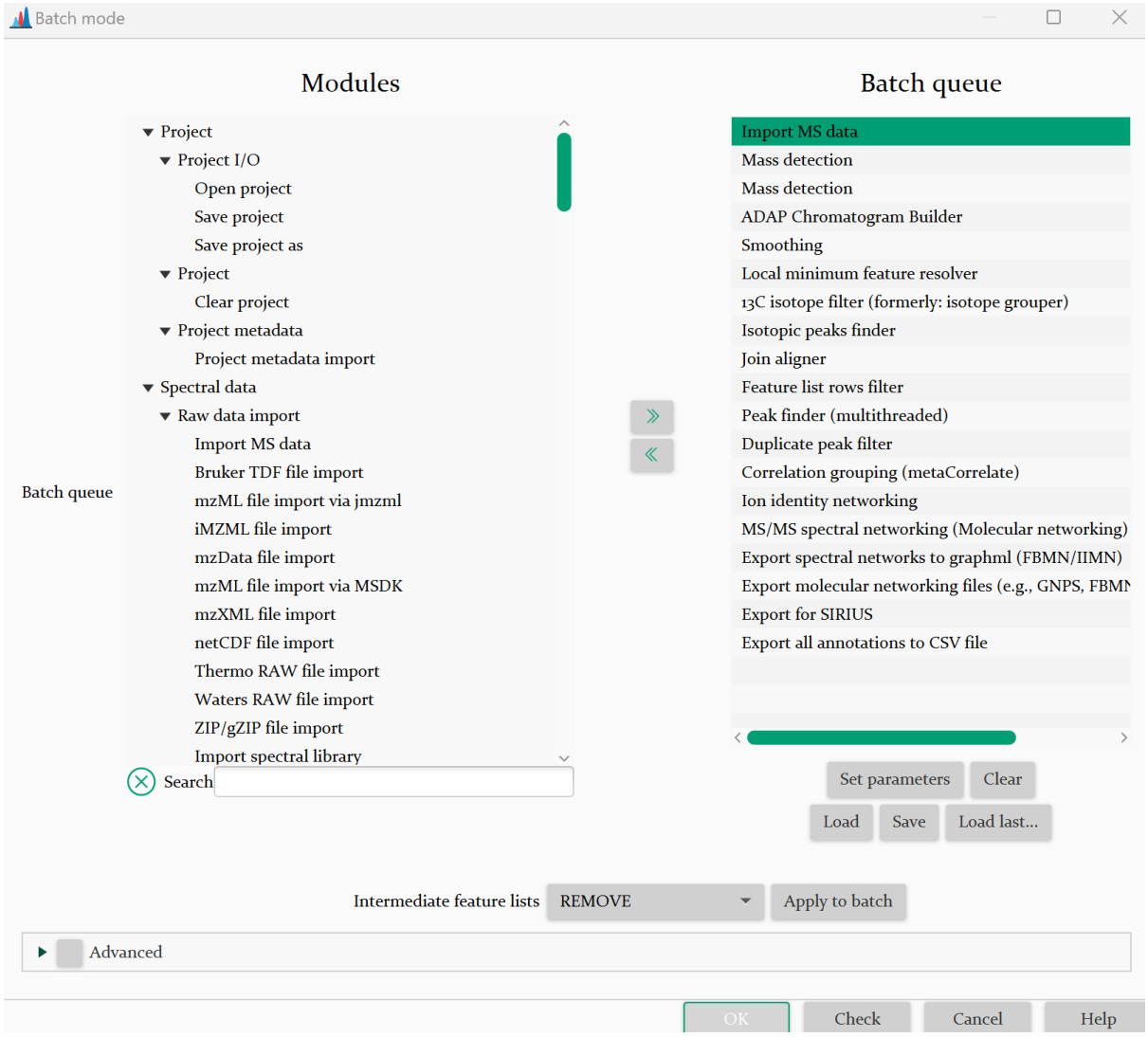
(c) Click ‘Processing wizard’.



(d) Set parameters in ‘HPLC’, ‘QTOF’, ‘Filters’, ‘Annotation’, and ‘DDA’. Basic explanation of parameters in Table S1. After setting, click ‘Create batch’.



(e) Set detailed parameter for each step (not necessary). Click 'OK' to process feature detection.



(f) Confirm the files with '.mgf' and ',csv' from mzmine.

Supplementary Figure 2. Basic form of metadata for GNPS.

Metadata in Excel has the following format. Subsequently, it is used by copying and pasting the content into a notepad file with the '.txt' extension. Each row represents individual files, while the columns indicate the categories of data to be written. Metadata sample download link: https://ccms-ucsd.github.io/GNPSDocumentation/metadata/ 'Barebones Metadata' part.

(a) Excel form of metadata.

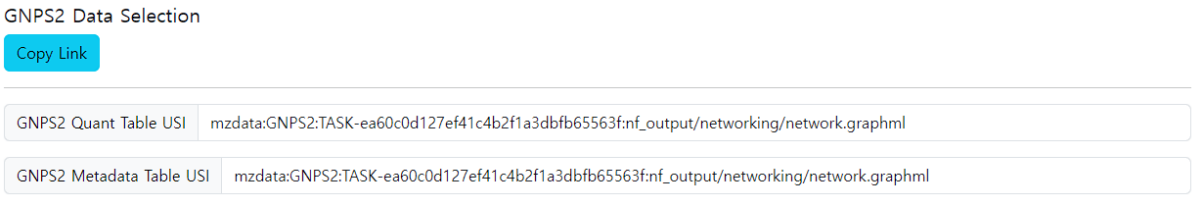
Filename	ATTRIBUTE_SampleType	ATTRIBUTE_SampleType2	ATTRIBUTE_Time	ATTRIBUTE_animal
s200525_Rat_Blank_01.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_Blank_02.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_Blank_03.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_4h_01.mzXML	sample	Invitro	4h	Rat
s200525_Rat_4h_02.mzXML	sample	Invitro	4h	Rat
s200525_Rat_4h_03.mzXML	sample	Invitro	4h	Rat
s200525_Mou_Blank_01.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_Blank_02.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_Blank_03.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_4h_01.mzXML	sample	Invitro	4h	Mouse
s200525_Mou_4h_02.mzXML	sample	Invitro	4h	Mouse
s200525_Mou_4h_03.mzXML	sample	Invitro	4h	Mouse
s190401_R2_30_01.mzXML	sample	Invivo	0.5h	Rat
s190401_R2_1h_01.mzXML	sample	Invivo	1h	Rat
s190401_R2_2h_01.mzXML	sample	Invivo	2h	Rat
s190401_R2_4h_01.mzXML	sample	Invivo	4h	Rat
s190401_R2_48h_01.mzXML	Blank	Invivo	48h	Rat
s190401_R1_30_01.mzXML	sample	Invivo	0.5h	Rat
s190401_R1_1h_01.mzXML	sample	Invivo	1h	Rat
s190401_R1_2h_01.mzXML	sample	Invivo	2h	Rat
s190401_R1_4h_01.mzXML	sample	Invivo	4h	Rat
s190401_R1_48h_01.mzXML	Blank	Invivo	48h	Rat
s190401_M3_30_01.mzXML	sample	Invivo	0.5h	Mouse

(b) Notepad file with '.txt' form of metadata.

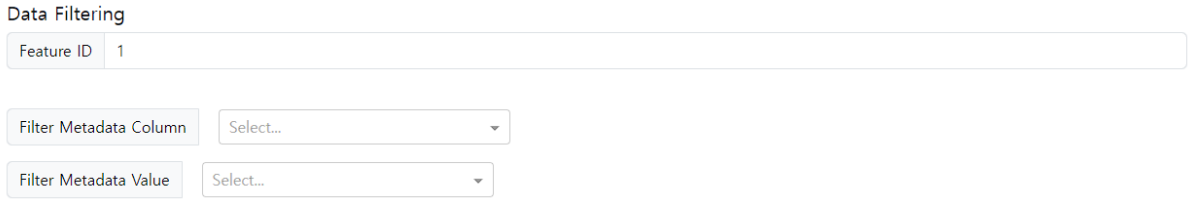
```
Filename	ATTRIBUTE_SampleType	ATTRIBUTE_SampleType2	ATTRIBUTE_Time	ATTRIBUTE_animal
s200525_Rat_Blank_01.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_Blank_02.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_Blank_03.mzXML	Blank	Invitro	4h	Rat
s200525_Rat_4h_01.mzXML	sample	Invitro	4h	Rat
s200525_Rat_4h_02.mzXML	sample	Invitro	4h	Rat
s200525_Rat_4h_03.mzXML	sample	Invitro	4h	Rat
s200525_Mou_Blank_01.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_Blank_02.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_Blank_03.mzXML	Blank	Invitro	4h	Mouse
s200525_Mou_4h_01.mzXML	sample	Invitro	4h	Mouse
s200525_Mou_4h_02.mzXML	sample	Invitro	4h	Mouse
s200525_Mou_4h_03.mzXML	sample	Invitro	4h	Mouse
s190401_R2_30_01.mzXML	sample	Invivo	0.5h	Rat
s190401_R2_1h_01.mzXML	sample	Invivo	1h	Rat
s190401_R2_2h_01.mzXML	sample	Invivo	2h	Rat
s190401_R2_4h_01.mzXML	sample	Invivo	4h	Rat
s190401_R2_48h_01.mzXML	Blank	Invivo	48h	Rat
s190401_R1_30_01.mzXML	sample	Invivo	0.5h	Rat
s190401_R1_1h_01.mzXML	sample	Invivo	1h	Rat
s190401_R1_2h_01.mzXML	sample	Invivo	2h	Rat
s190401_R1_4h_01.mzXML	sample	Invivo	4h	Rat
s190401_R1_48h_01.mzXML	Blank	Invivo	48h	Rat
s190401_M3_30_01.mzXML	sample	Invivo	0.5h	Mouse
s190401_M3_1h_01.mzXML	sample	Invivo	1h	Mouse
s190401_M3_2h_01.mzXML	sample	Invivo	2h	Mouse
s190401_M3_4h_01.mzXML	sample	Invivo	4h	Mouse
s190401_M3_48h_01.mzXML	Blank	Invivo	48h	Mouse
s190401_M2_30_01.mzXML	sample	Invivo	0.5h	Mouse
s190401_M2_1h_01.mzXML	sample	Invivo	1h	Mouse
s190401_M2_2h_01.mzXML	sample	Invivo	2h	Mouse
s190401_M2_4h_01.mzXML	sample	Invivo	4h	Mouse
s190401_M2_48h_01.mzXML	Blank	Invivo	48h	Mouse
```

Supplementary Figure 3. Step-by-step screenshots of Workflow #2, quantitative analysis, at website (https://plotter.gnps2.org/).

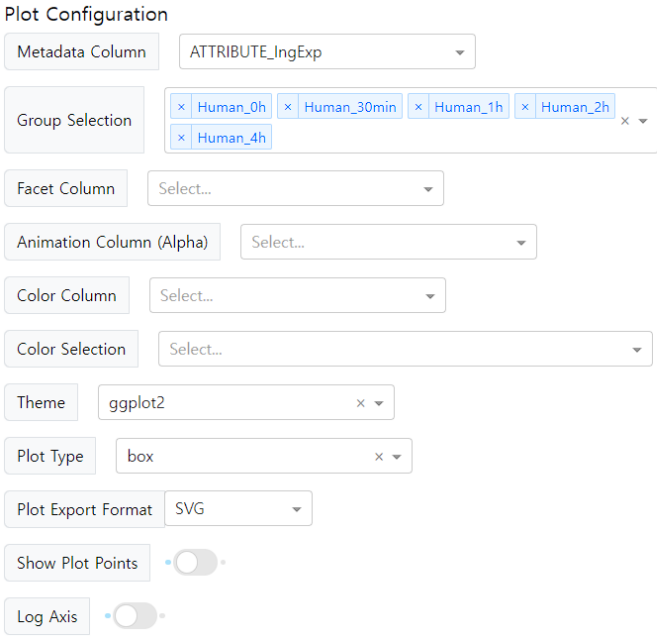
(a) Fill USI.



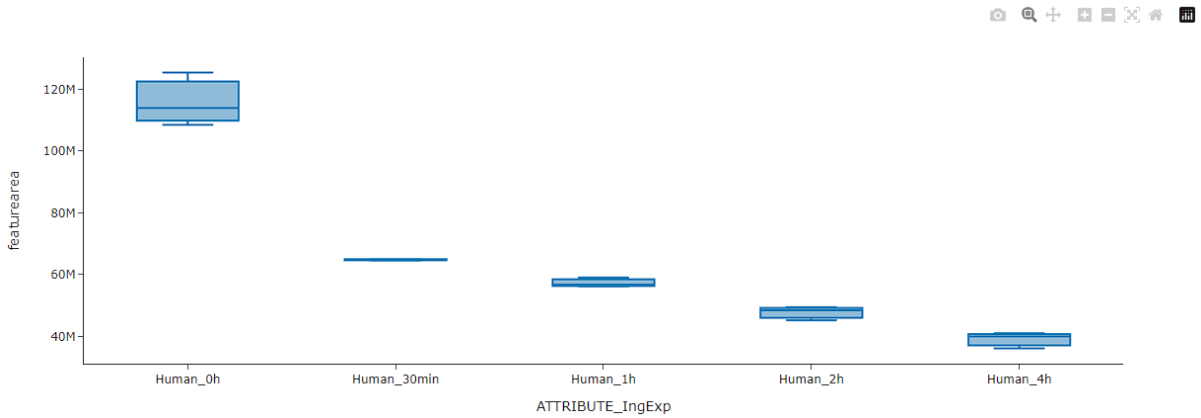
(b) Fill node ID.



(c) Set parameters.

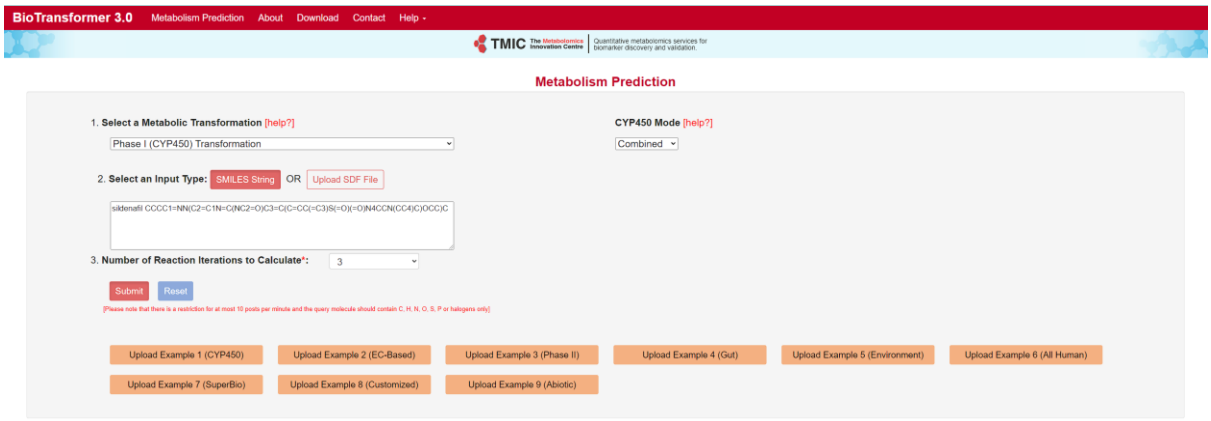


(d) Box plot.


120M
100M
80M
60M
40M
featurearea
Human_0h
Human_30min
Human_1h
Human_2h
Human_4h
ATTRIBUTE_IngExp

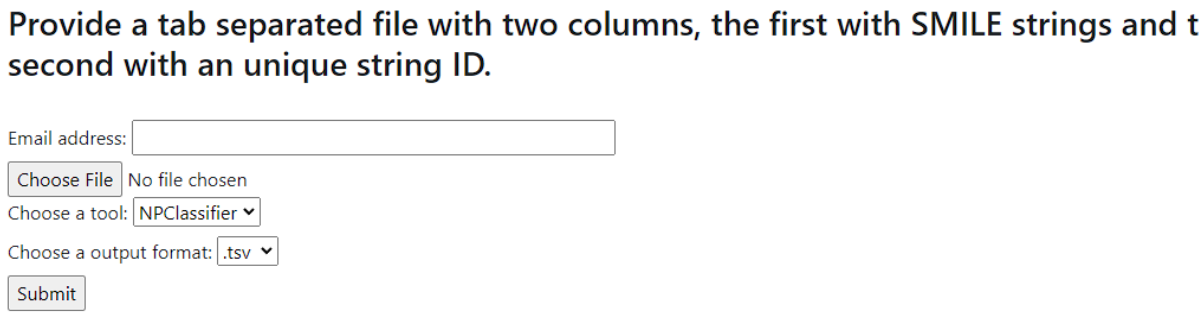
Supplementary Figure 4. Step-by-step screenshots of Workflow #3, Metabolite structure prediction.

(a) Make metabolites list by Biotransformer 3.0.

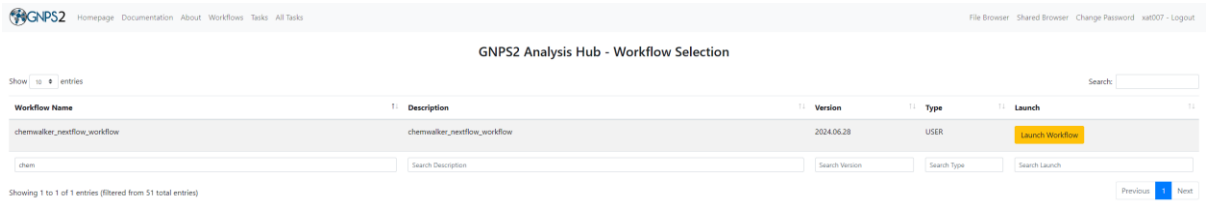


	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Result_ID	BTMR_ID	Metabolit	Synonyms	PUBCHEM	InChI	InChIKey	SMILES	Molecular	Major_Isot	ALogP	Biosystem	Enzyme(s)	Reaction	Precursor_	Precursor_	Precursor_	Precursor_	Precursor_
2	1	BTMR0941	BT00001			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	490.1998	2.0882	Human	CYP1A2; C	EAWAG_R	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
3	2	BTMR0042	BT00002	Desethyl S	1.36E+08	InChI=1S/	InChIKey=	CCCC1=NC	C20H26N6	446.1736	2.1689	Human	CYP1A2; C	N-Hydroxy	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
4	3	BTMR0042	BT00003	acetaldehy	177	InChI=1S/	InChIKey=	CC=O	C2H4O	44.02621	-0.1825	Human	CYP1A2; C	N-Hydroxy	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
5	4	BTMR1148	BT00004	Sildenafil	1.36E+08	InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	490.1998	2.3636	Human	CYP1A2; C	N-Dealkyl	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
6	5	BTMR9031	BT00005	N-Desmet	1.35E+08	InChI=1S/	InChIKey=	CCCC1=NC	C21H28N6	460.1892	2.2328	Human	CYP1A2; C	1-Naphth	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
7	6	BTMR1354	BT00006			InChI=1S/	InChIKey=	CC(CC1=N	C22H30N6	490.1998	1.5993	Human	CYP1A2; C	Hydrolysis	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
8	7	BTMR1349	BT00007			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	490.1998	2.429	Human	CYP1A2; C	Hydrolytic	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
9	8	BTMR1349	BT00008			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	490.1998	2.429	Human	CYP1A2; C	Hydrolytic	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
10	9	BTMR1317	BT00009	SCHEMBL	1.36E+08	InChI=1S/	InChIKey=	C(CCC1=N	C22H30N6	490.1998	1.543	Human	CYP1A2; C	N-Glucuro	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
11	10	BTMR1317	BT00010			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	490.1998	1.8805	Human	CYP1A2; C	N-Glucuro	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
12	11	BTMR0505	BT00011			InChI=1S/	InChIKey=	C=CCC1=	C22H28N6	472.1892	2.378	Human	CYP1A2; C	7-OH-Sulf	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
13	12	BTMR1077	BT00012			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	490.1998	2.0408	Human	CYP3A4	Hydroxyla	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
14	13	BTMR1292	BT00013	Pyrazole N	1.36E+08	InChI=1S/	InChIKey=	CCCC1=NC	C21H28N6	460.1893	2.5628	Human	CYP2D6; C	Isoflavano	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
15	14	BTMR1292	BT00014	formaldeh	712	InChI=1S/	InChIKey=	O=C	CH2O	30.01056	-0.8841	Human	CYP2C9; C	Isoflavano	sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.7687
16	15	BTMR0079	BT00015			InChI=1S/	InChIKey=	CCC(C1=N	C22H28N6	488.1841	2.1983	Human	CYP1A2; C	Oxidation	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
17	16	BTMR0052	BT00016			InChI=1S/	InChIKey=	CCC(C1=N	C20H26N6	462.1685	1.4884	Human	CYP1A2; C	O-Dealkyl	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
18	17	BTMR0052	BT00003	acetaldehy	177	InChI=1S/	InChIKey=	CC=O	C2H4O	44.02621	-0.1825	Human	CYP1A2; C	O-Dealkyl	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
19	18	BTMR1099	BT00017			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	506.1947	1.6831	Human	CYP1A2; C	N-Oxidati	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
20	19	BTMR9285	BT00018			InChI=1S/	InChIKey=	CCC(C1=N	C21H28N6	476.1841	1.5523	Human	CYP1A2; C	N-Dealkyl	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
21	20	BTMR9320	BT00019			InChI=1S/	InChIKey=	CC(C(C1=	C22H30N6	506.1947	1.0538	Human	CYP1A2; C	Hydroxyla	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
22	21	BTMR1070	BT00020			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	506.1947	1.7485	Human	CYP1A2; C	Hydroxyla	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
23	22	BTMR1070	BT00021			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	506.1947	1.7485	Human	CYP1A2; C	Hydroxyla	sildenafil	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
24	23	BTMR9285	BT00022			InChI=1S/	InChIKey=	C(CC(C1=	C22H30N6	506.1947	0.7387	Human	CYP1A2; C	Hydroxyla	sildenafil	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
25	24	BTMR9285	BT00023			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	506.1947	1.2	Human	CYP1A2; C	Hydroxyla	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
26	25	BTMR1190	BT00024			InChI=1S/	InChIKey=	C=CC(C1=	C22H28N6	488.1841	1.8325	Human	CYP1A2; C	Terminal c	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
27	26	BTMR1328	BT00025			InChI=1S/	InChIKey=	CCC(C1=N	C22H30N6	506.1948	1.3603	Human	CYP3A4	Hydroxyla	sildenafil	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
28	27	BTMR1332	BT00026			InChI=1S/	InChIKey=	CCC(C1=N	C21H28N6	476.1842	1.8823	Human	CYP2D6; C	N-Dealkyl	InChIKey=	InChI=1S/	InChIKey=	CCC(C1=N	2.0882
29	28	BTMR1099	BT00027			InChI=1S/	InChIKey=	CCCC1=NC	C20H26N6	462.1685	1.7638	Human	CYP1A2; C	N-Oxidati	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
30	29	BTMR9285	BT00028			InChI=1S/	InChIKey=	CCCC1=NC	C19H24N6	432.1579	1.633	Human	CYP1A2; C	N-Dealkyl	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
31	30	BTMR9320	BT00029			InChI=1S/	InChIKey=	CC(CC1=N	C20H26N6	462.1685	0.999499	Human	CYP1A2; C	Hydroxyla	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
32	31	BTMR1070	BT00030			InChI=1S/	InChIKey=	CCCC1=NC	C20H26N6	462.1685	1.8292	Human	CYP1A2; C	Hydroxyla	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
33	32	BTMR1070	BT00031			InChI=1S/	InChIKey=	CCCC1=NC	C20H26N6	462.1685	1.8292	Human	CYP1A2; C	Hydroxyla	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
34	33	BTMR9285	BT00032			InChI=1S/	InChIKey=	C(CCC1=N	C20H26N6	462.1685	0.9432	Human	CYP1A2; C	Hydroxyla	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
35	34	BTMR1190	BT00033			InChI=1S/	InChIKey=	C=CCC1=	C20H24N6	444.1579	1.7782	Human	CYP1A2; C	Terminal c	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
36	35	BTMR1037	BT00034			InChI=1S/	InChIKey=	CCCC1=NC	C20H26N6	462.1685	1.9015	Human	CYP1A2; C	O-Hydroxy	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
37	36	BTMR1332	BT00035			InChI=1S/	InChIKey=	CCCC1=NC	C19H24N6	432.158	1.963	Human	CYP1A2; C	N-Dealkyl	Desethyl S	InChI=1S/	InChIKey=	CCCC1=N	2.1689
38	37	BTMR0862	BT00036	hydron;ac	21980959	InChI=1S/	InChIKey=	CC(=O)O	C2H4O2	60.02112	-0.2299	Human	CYP1A2; C	EAWAG_R	acetaldehy	InChI=1S/	InChIKey=	CC=O	-0.1825
39	38	BTMR1277	BT00037	glycolalde	756	InChI=1S/	InChIKey=	C(C=O)O	C2H4O2	60.02112	-0.64109	Human	CYP1A2; C	alpha-Hyd	acetaldehy	InChI=1S/	InChIKey=	CC=O	-0.1825
40	39	BTMR0945	BT00038	ethanol; e	702	InChI=1S/	InChIKey=	CCO	C2H6O	46.04186	-0.00919	Human	CYP3A4	EAWAG_R	acetaldehy	InChI=1S/	InChIKey=	CC=O	-0.1825
41	40	BTMR9320	BT00039			InChI=1S/	InChIKey=	CC(CC1=N	C22H30N6	506.1947	1.1942	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
42	41	BTMR9290	BT00040			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	506.1947	2.0239	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
43	42	BTMR9290	BT00041			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	506.1947	2.0239	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
44	43	BTMR1061	BT00042			InChI=1S/	InChIKey=	C(CCC1=N	C22H30N6	506.1947	1.1379	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
45	44	BTMR1061	BT00043			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	506.1947	1.4754	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
46	45	BTMR1190	BT00044			InChI=1S/	InChIKey=	C=CCC1=	C22H28N6	488.1841	1.9729	Human	CYP1A2; C	Terminal c	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
47	46	BTMR1328	BT00045			InChI=1S/	InChIKey=	CCCC1=NC	C22H30N6	506.1948	1.6357	Human	CYP1A2; C	Hydroxyla	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636
48	47	BTMR1332	BT00046			InChI=1S/	InChIKey=	CCCC1=NC	C21H27N	475.1764	1.8723	Human	CYP1A2; C	N-Dealkyl	Sildenafil	InChI=1S/	InChIKey=	CCCC1=N	2.3636

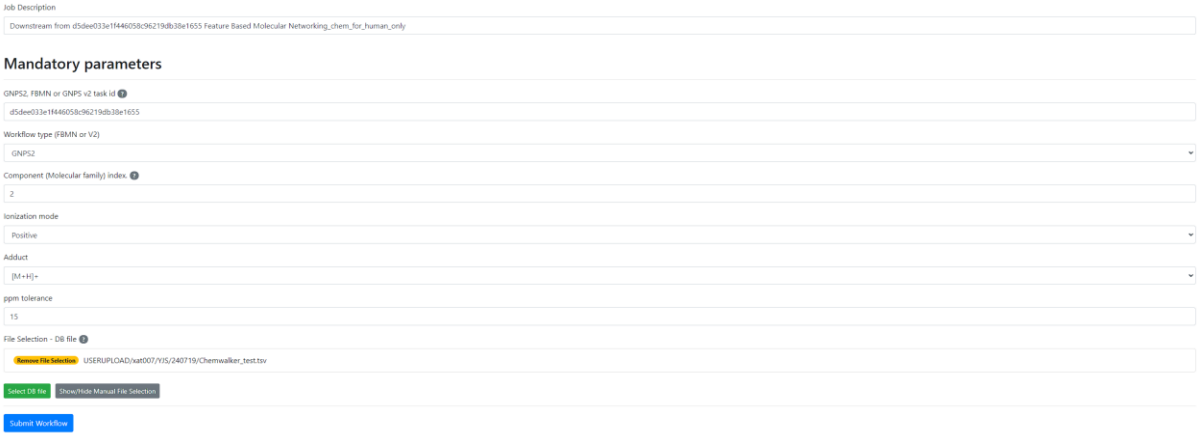
(b) Database generation through NPClassifier at http://seriema.fcfrp.usp.br:5002/upload.



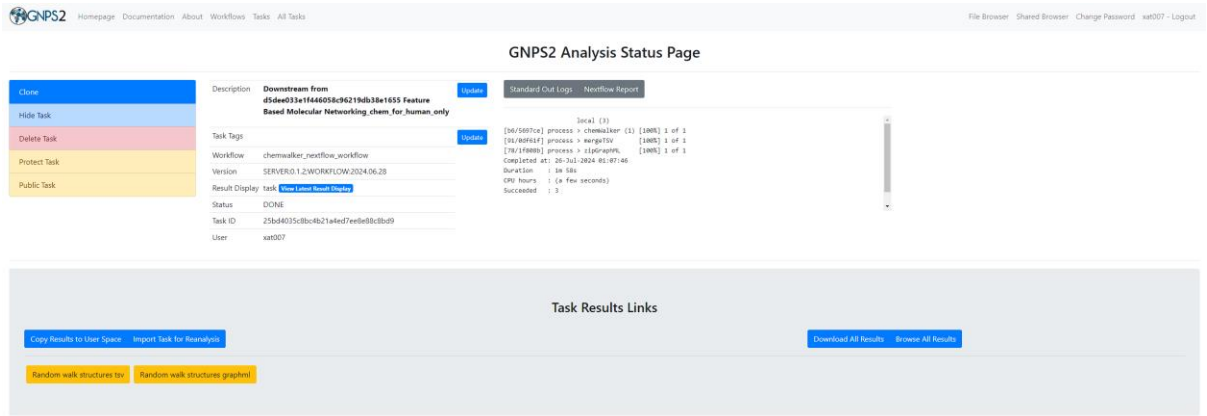
(c) Upload database file and start the chemwalker.



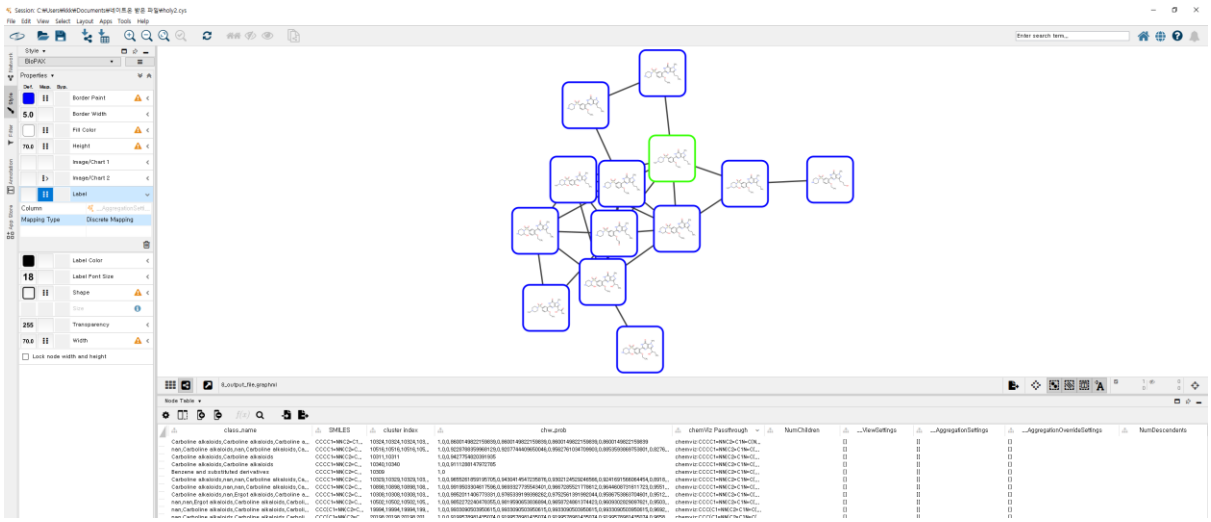
(d) Set parameters.



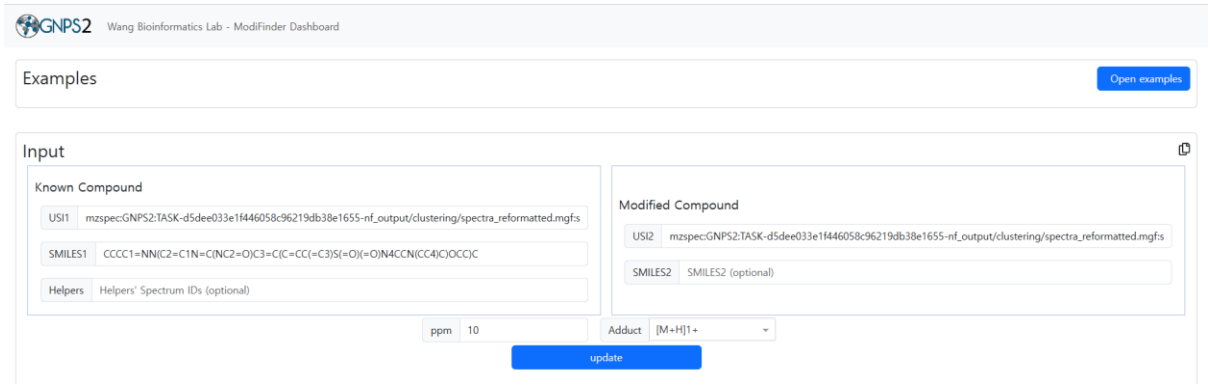
(e) Submit workflow, download ‘Random walk structures graphml’.



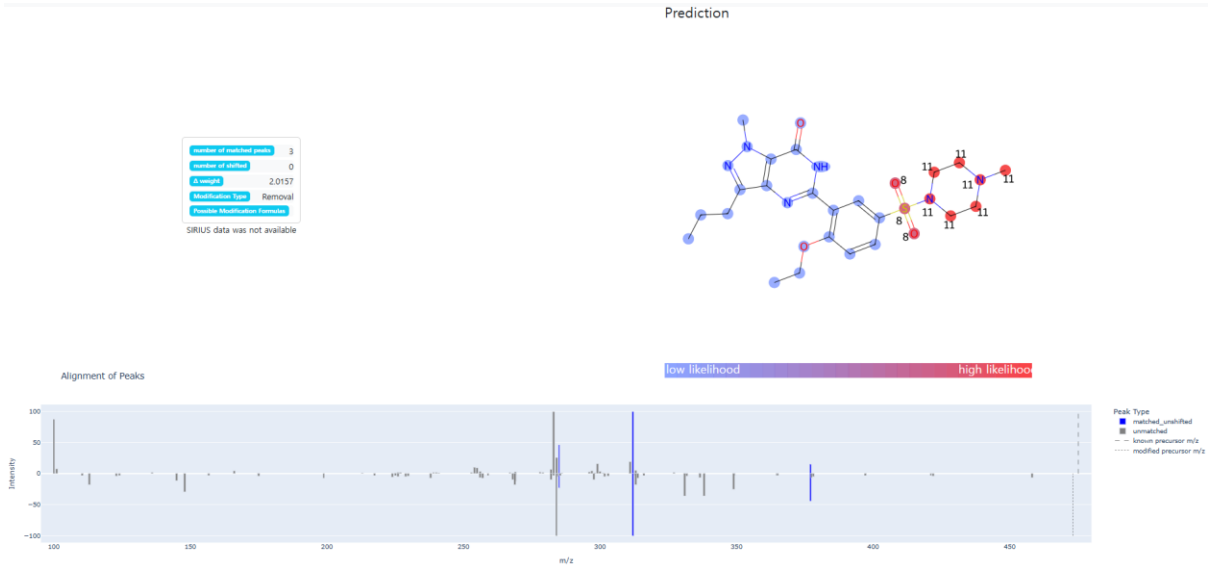
(f) Visualization in cytoscape.



(g) Go to Modifinder (https://modifinder.gnps2.org) and input USI and SMILES.



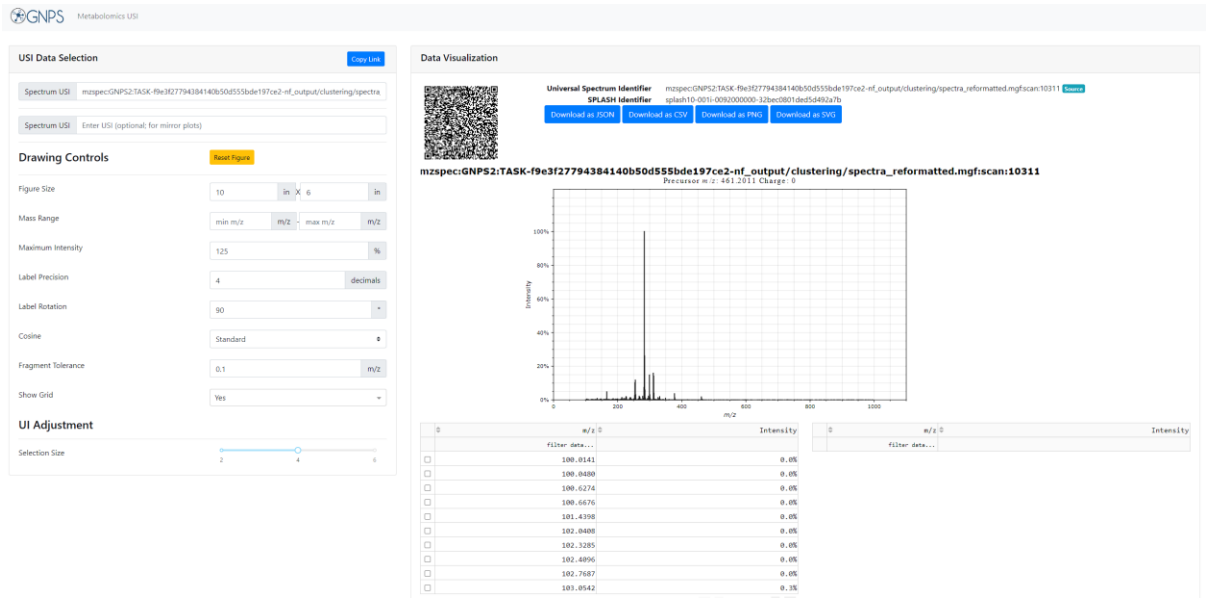
(h) Confirm the results and curate structures of metabolites.



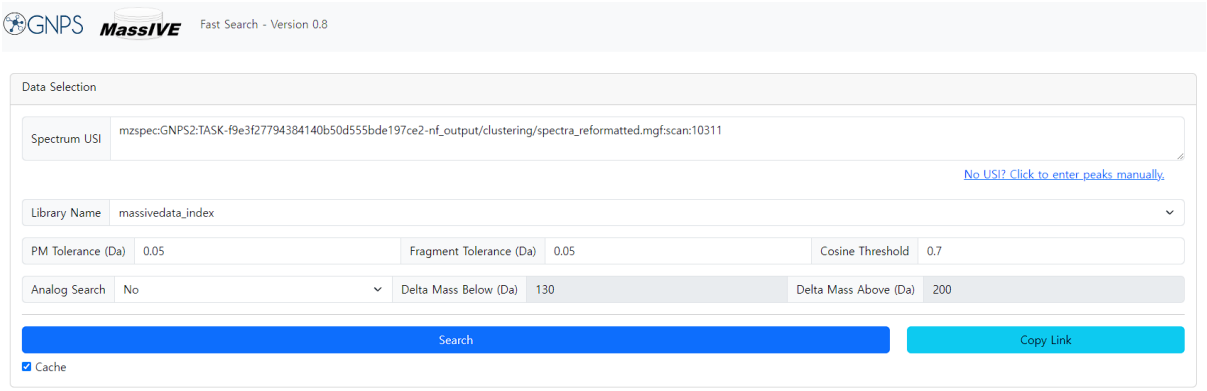
Supplementary Figure 5. Step-by-step screenshots of Workflow #5, Repository search.

(a) In FBMN, click the interested metabolite node and go to 'View Spectrum USI'.

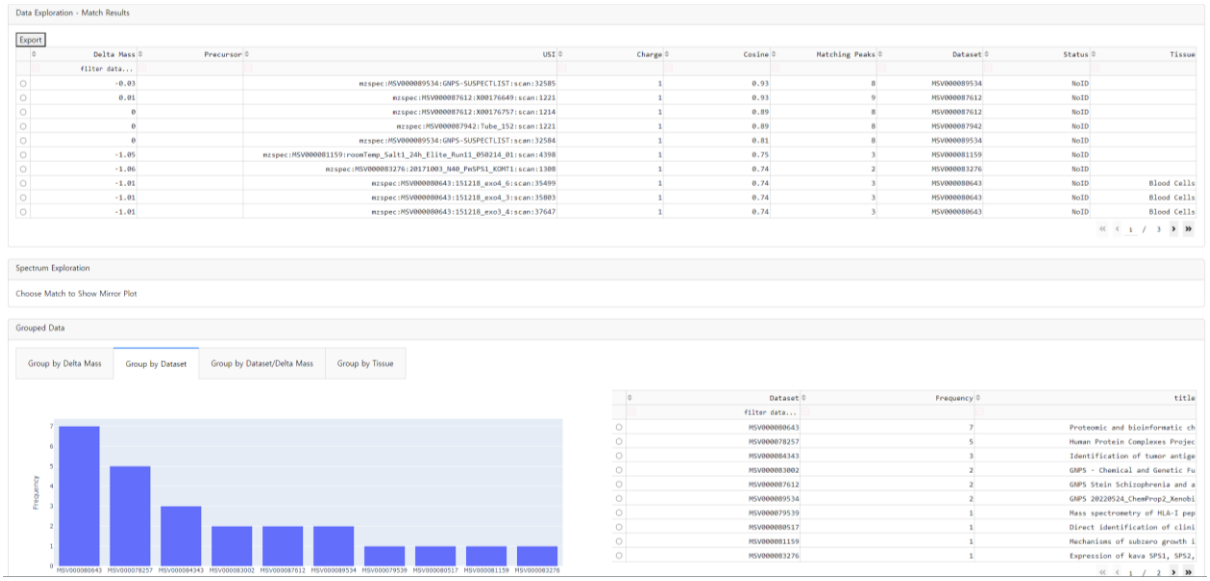
(b) Confirm the spectrum USI and copy it.



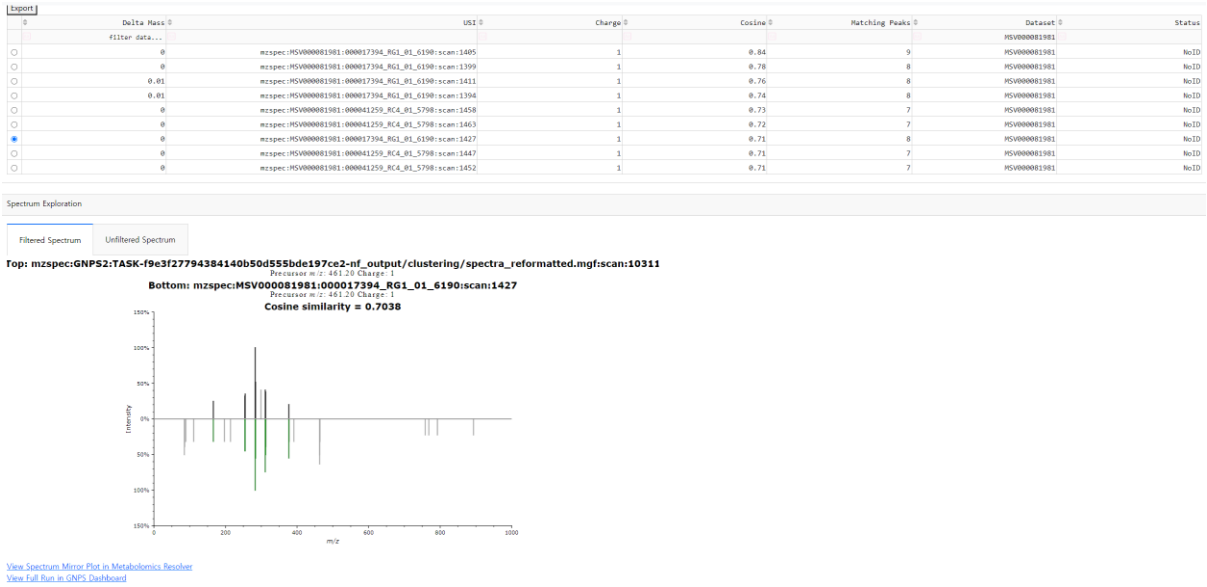
(c) Go to Fast search and fill the spectrum USI.



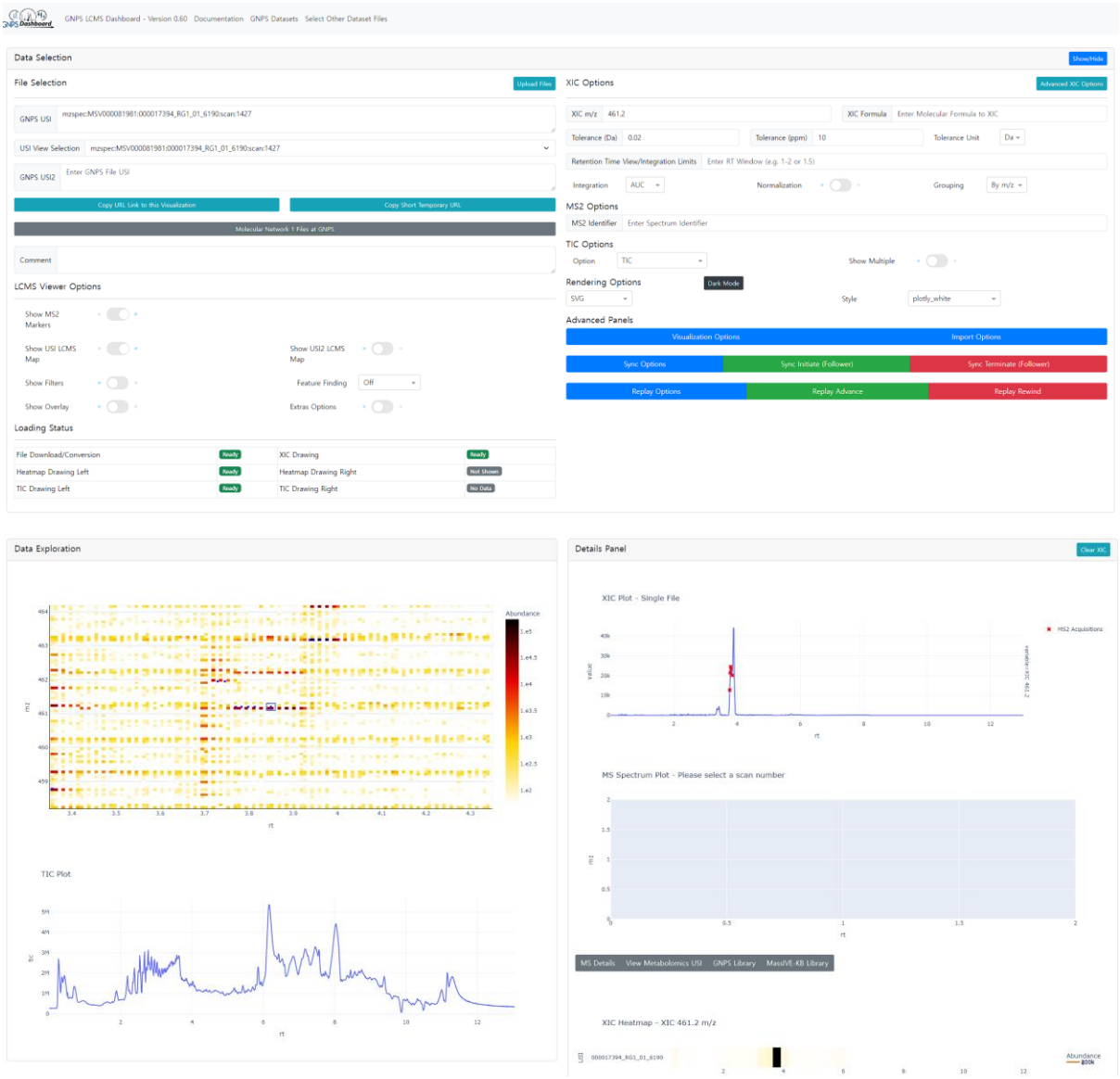
(d) Search and confirm the data matched.



(e) Confirm the MS/MS data of interested datasets.



(f) Confirm the MS data through GNPS LCMS Dashboard by link, ‘View Full Run in GNPS Dashboard’.



Supplementary Tables

Supplementary Table 1. Feature detection parameters in mzmine 'Processing wizard'.

Available parameters	Definition	Recommended input
HPLC		
Smoothing	Apply smoothing in the retention time dimension.	Check
Stable ionization across sample	Ensure stable ionization conditions across all samples	Check
Crop retention time	Crops the RT range of chromatograms	Setting with 'Auto range'
Max peaks in chromatogram	An estimate maximum number of peaks (same m/z)	Default '15'
Minimum consecutive scans	Minimum number of scan points with detected data used in chromatogram building	More than three.
Approximate feature FWHM	The approximate feature width in retention time	The settings can vary depending on the peak shape. Here, 0.1 min was used
RT tolerance	RT tolerance for multiple signals of the same compound in the same or different samples	The settings can vary depending on the shape and reproducibility between peaks. In this case, intra-sample was set to 0.1 min, and sample-to-sample to 0.2 min
QTOF		
Ion mode	Polarity of ion mode	Positive or Negative
Noise threshold	Noise level for analysis	Choose appropriately based on peak sensitivity. Here, Absolute intensity MS1: 500-1000, MS2: 50-100 was selected.
Minimum feature height	Intensity threshold to be recognized as a feature	Select based on peak sensitivity. Here, a range of 1000-2000 was chosen
m/z tolerance	The m/z fluctuations of peaks from one scan to another within same scans, samples or different samples	For GNPS, QTOF m/z tolerance was set to 0.02 based on their recommendation (0.02, 10 ppm). For higher resolution, smaller values can be used and adjusted as needed.
Filters		
Original feature list	Defines the processing	Default 'REMOVE' saves memory. If errors occur and you need to identify the

		problematic step, select 'KEEP' to confirm errors
Min samples per aligned feature	The minimum number of samples in which a feature needs to be detected.	This depends on the number of replicates for the analyzed data (e.g., 2-3 for triplicates).
Only keep features with 13C	Filter out peaks that have no feature with a 13C isotope pattern	Not check
Annotation		
Local CSV database	Sections for additional annotation to aid networking information are available but not used in this study.	
DDA		
Apply spectral networking (FBMN/IIMN)	Applies FBMN/IIMN by comparing all MS2 spectra	MZmine independently performs FBMN and provides data for Cytoscape (.graphml). This is checked but not utilized.
Export path	Specify the file path for extracted data as needed	Check and enter appropriately depending on the situation (e.g., C:\Yu\2025\NP\)
Export for molecular networking (e.g. GNPS, FBMN, IIMN, MetGem)	Create a '.mgf' file for FBMN in GNPS	Check
Export for SIRIUS	Generate files for applying SIRIUS	Not check
Export annotation graphics	Create image files for annotation	Not check

Supplementary Table 2. FBMN parameters in these protocols.

Parameter	Value
featurefindingtool	MZMINE
fragment_tolerance	0.02
library_analog_search	0
library_min_cosine	0.7
library_min_matched_peaks	6
library_topk	1
min_peak_intensity	0
networking_max_shift	1999
networking_min_cosine	0.5
networking_min_matched_peaks	6
normalization	None
pm_tolerance	0.02
precursor_filter	Yes/No
window_filter	No

Supplementary Table 3. Metabolite node list in feature-based molecular networking of comparative metabolism study.

Metabolites	m/z	Retention time (min)
Sildenafil	475.2161	17.29
M377	377.1309	12.43
M392	392.1482	20.72
M407	407.1552	14.70
M421a	421.1667	5.29
M421b	421.1699	15.37
M422	422.1528	11.34
M433	433.1801	18.51
M435a	435.1828	5.68
M435b	435.1841	11.82
M436	436.1688	21.69
M447	447.1825	9.21
M449a	449.1986	12.50
M449b	449.1658	20.02
M450	450.1473	23.27
M461a	461.1987	9.24
M461b	461.2011	17.18
M463	463.1786	20.93
M475a	475.1805	23.44
M475b	475.1795	26.67
M477a	477.1941	10.01
M477d	477.1952	24.04
M477e	477.1933	26.14
M491c	491.2104	18.72
M491d	491.1774	21.81
M491e	491.1780	22.40
M493a	493.1907	13.66
M493b	493.1916	19.52
M505a	505.1892	25.56
M509	509.1854	18.07
M513	513.1907	18.71
M540	540.2046	13.05

Supplementary Table 4. Metabolite nodes list in feature-based molecular networking of CYP reaction phenotyping study.

Metabolites	m/z	Retention time (min)
Sildenafil	475.2151	13.98
M377	377.1363	9.35
M435a	435.1917	9.41
M436	436.1676	17.39
M449a	449.2033	10.00
M461b	461.2021	13.86
M463	463.1847	16.69
M475b	475.1824	21.58
M477b	477.2104	18.88
M477c	477.2041	19.29
M477d	477.2102	19.60
M477e	477.2046	21.22
M489a	489.2045	4.99
M489b	489.2034	24.89
M491a	491.2123	4.29
M491b	491.2143	4.80
M491c	491.2107	15.34

Supplementary Table 5. Metabolite nodes list in feature-based molecular networking of *in vivo* metabolism study of mice.

Metabolites	m/z	Retention time (min)
Sildenafil	475.2128	16.01
M377	377.1276	11.08
M392	392.1387	19.36
M435a	435.1821	10.71
M436	436.1652	20.15
M449a	449.2103	10.83
M459	459.1813	15.78
M461b	461.1962	15.84
M473	473.1964	16.90
M475b	475.1761	24.97
M477e	477.1931	24.39
M489b	489.1928	28.52
M491c	491.2082	17.36

Supplementary Methods

Chemicals

Sildenafil citrate was purchased from Sigma Aldrich (St. Louis, MO, USA). Pooled human, rat, and mouse liver microsomes were obtained from BD Gentest (Woburn, MA, USA), and pooled monkey and dog liver microsomes were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA). Glucose-6-phosphate, β-$NADP^+$, and glucose-6-phosphate dehydrogenase were obtained from Milliporen Sigma (St. Louis, MO, USA). All other chemicals used in this article were of analytical grade and were used as received.

In Vitro Biotransformation of Sildenafil

Sildenafil citrate at a final concentration of 25 μM and was incubated with 1 mg/mL microsomal protein from human liver in 0.1 M potassium phosphate buffer (pH 7.4) at 37 ℃ in a final incubation volume of 200 μL. The reaction was initiated by the addition of an NADPH-generating system (NGS) containing 0.8 mM β-$NADP^+$, 10 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase to the reaction mixture. Reaction control samples (named No NGS) were prepared by using distilled water instead of NGS, and blank samples were prepared by putting DMSO instead of sildenafil citrate. For inter-species sildenafil biotransformation, microsomal protein from the corresponding animal species liver was used instead of human liver. In reaction phenotyping, supersomes for CYP1A2, 2A6, 2C8, 2C9, 2D6, 2E1, 2J2, 3A4, and 3A5 were used instead of human liver microsomes. After incubation, 50 μL of MeCN with 1% formic acid was added, then the solution was centrifuged at 13,200 rpm. 5 μL of supernatants were acquired for LC–MS analyses. Every biotransformation was triplicated. No unexpected or unusually high safety hazards were encountered.

In Vivo Biotransformation of Sildenafil at mice

Male C57BL/6 mice (8-week-old, weighing approximately 22–25 g) were purchased from Orient Bio (Songnam, Korea). The mice were housed in a temperature-controlled (23 ± 2°C), humidity-controlled (55 ± 10%) room with a 12-hour light/dark cycle and had ad libitum access to food and water. Sildenafil citrate was dissolved in distilled water, and the mice were orally administered a solution of sildenafil citrate (20 mg/kg). Blood samples were collected from the orbital vein into heparinized tubes at 0.5, 1, 2, and 4 hours after oral administration. The blood samples were centrifuged to isolate plasma, and 25 μL aliquots of plasma from each sample were stored at −20°C until analysis. The plasma samples were precipitated with MeCN (50 μL) and vortexed for 1 minute. After centrifugation at 13,200 rpm for 10 minutes, a 30 μL aliquot of the supernatant was transferred to an autosampler, and 5 μL was injected into the LC-QTOF/MS system. All animal procedures were approved by the Institutional Animal Care and Use Committee of Hanyang University (2017-0023).

Data Acquisition

The samples were analyzed using an Agilent 6530 quadrupole time-of-flight (Q/TOF) mass spectrometer coupled with an Agilent 1260 HPLC system (Agilent Technologies, Inc., Santa Clara, CA,

USA). Chromatographic separation was performed with a Hypersil GOLD C_{18} column (2.1 mm × 150 mm, 3 µM, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) maintained at 40 ℃. The mobile phase consisted of water (A) and 90% MeCN (B), both of which were acidified with 0.1% formic acid. The mobile phase was eluted at a flow rate of 0.25 mL/min, with a linear gradient from 20% to 35% B for 30 min, followed by 4 min of washing with 90% B and 9 min of re-conditioning with 20% B. The entire column eluent was directly introduced into an electrospray ionization interface. Nitrogen was used both as the nebulizing gas at 20 psi and as the drying gas with a flow rate of 10 L/min at a temperature of 300 ℃. The mass spectrometer was operated in the positive ion mode and data-dependent acquisition with MS and MS/MS ranges of *m/z* 300–1000 and 100–1000, respectively. The collision energy of fragmentation was 43 eV. Helium was used as collision gas.