

1 **Lipidomic analyses uncover apoptotic and inhibitory**
2 **effects of pyrvinium pamoate on cholangiocarcinoma**
3 **cells via mitochondrial membrane potential**
4 **dysfunction**

5
6 **Yingpinyapat Kittirat^{1,2}, Jutarop Phetcharaburanin^{1,2,3}, Bundit Promraksa^{1,2},**
7 **Thanaporn Kultawatsiri^{1,2,3}, Arporn Wangwiwatsin^{1,2,3}, Poramate Klanrit^{1,2,3},**
8 **Prakasit Sa-Ngiamwibool^{2,4}, Apiwat Jarearnrat^{2,5}, Suyanee Thongchot^{6,7}, Panupong**
9 **Mahalabutr^{1,2,3}, Watcharin Loilome^{1,2,3} Jia V. Li^{8,9}, Hideyuki Saya¹⁰, Nisana**
10 **Namwat^{1,2,3*}**

11
12 ¹Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

13 ²Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002,
14 Thailand

15 ³Khon Kaen University International Phenome Laboratory, Khon Kaen University Science Park, Innovation
16 and Enterprise Affairs, Khon Kaen University, Khon Kaen, 40002, Thailand

17 ⁴Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

18 ⁵Department of Surgery, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

19 ⁶Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700,
20 Thailand

21 ⁷Siriraj Center of Research Excellence for Cancer Immunotherapy, Faculty of Medicine Siriraj Hospital,
22 Mahidol University 10700 Thailand

23 ⁸Department of Metabolism, Digestive Disease and Reproduction, Faculty of Medicine, Imperial College
24 London, South Kensington, London SW7 2AZ, UK

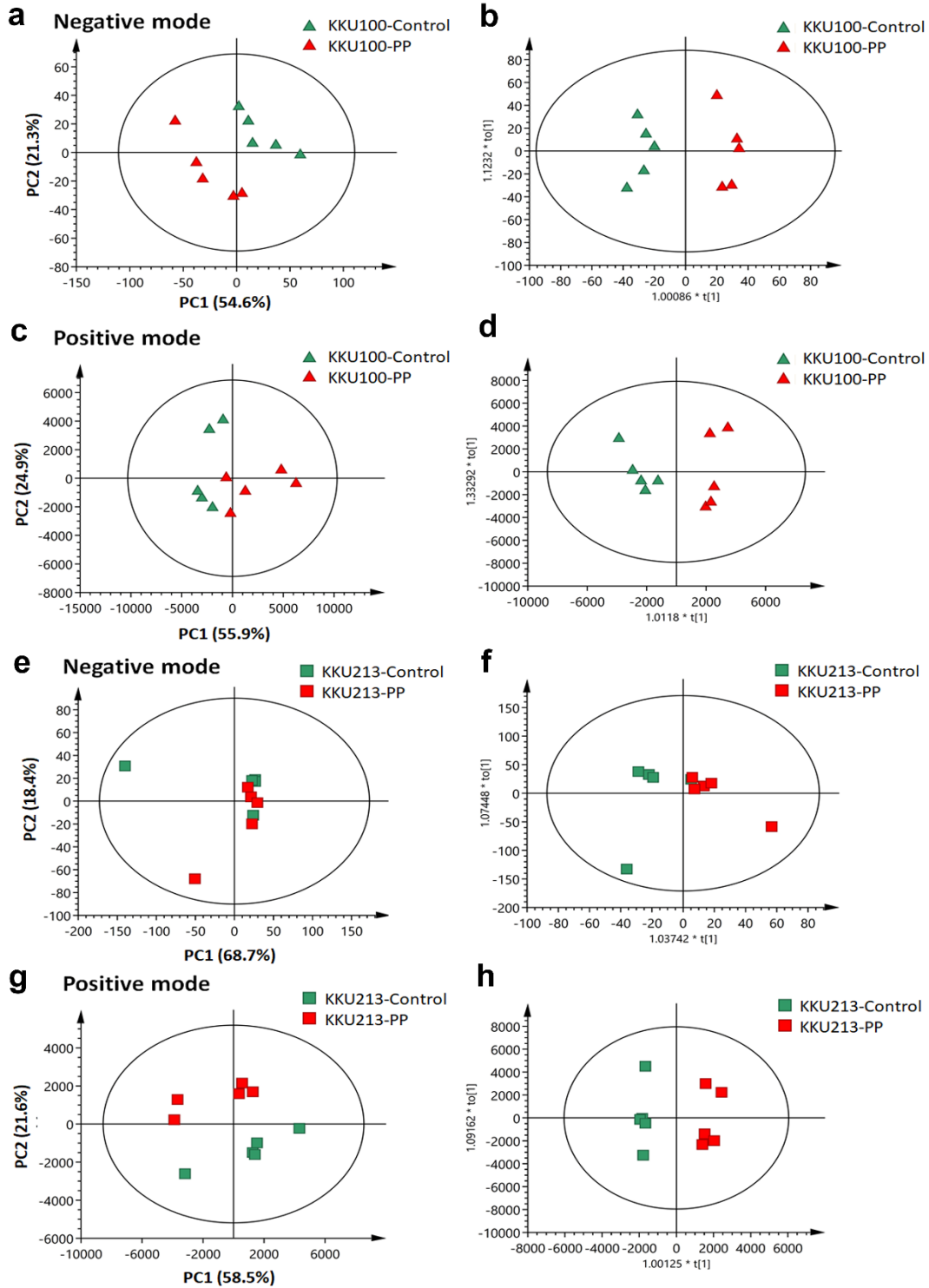
25 ⁹Centre for Digestive and Gut Health, Institute of Global Health Innovation, Imperial College London,
26 London SW7 2AZ, UK

27 ¹⁰Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio
28 University, Tokyo, Japan

29
30 *Corresponding author

31 Assoc. Prof. Nisana Namwat, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, 123
32 Mittraphap Road, Nai-Muang, Muang District, Khon Kaen 40002, Thailand

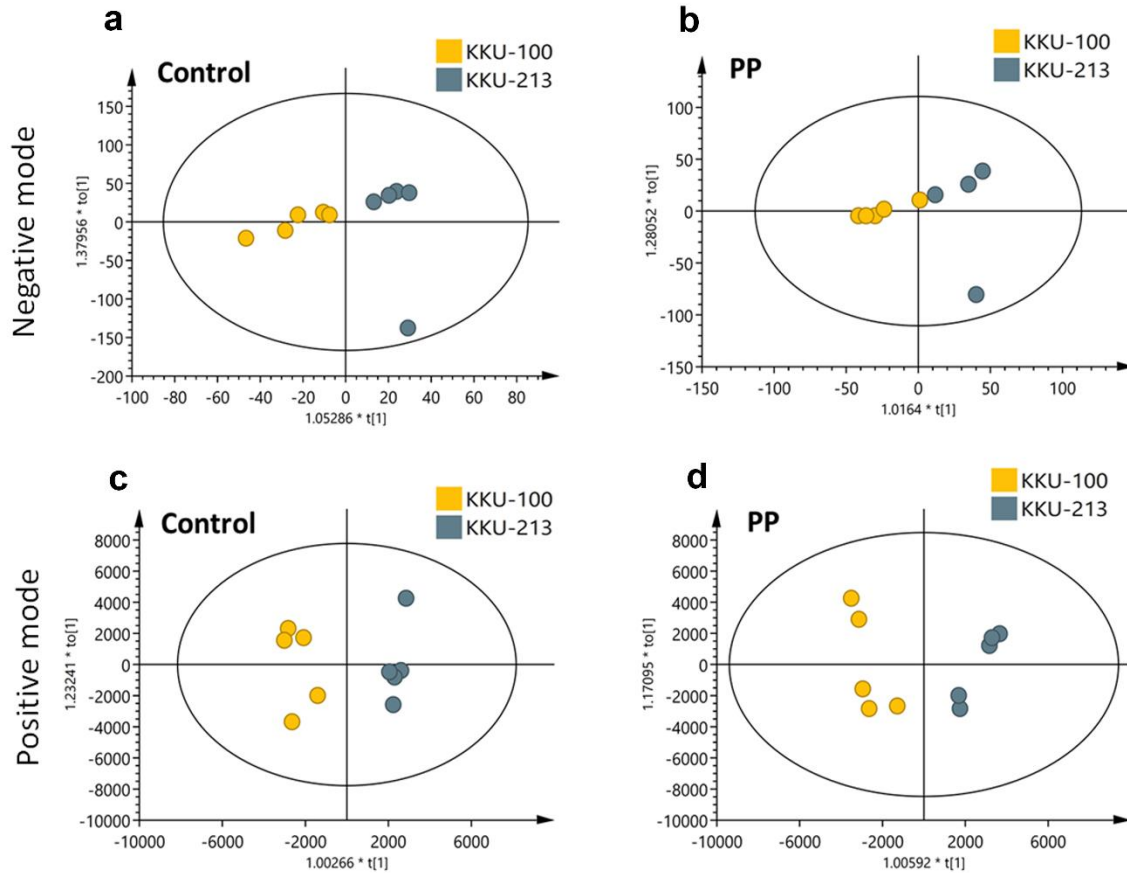
33 E-mail: nisana@kku.ac.th



35

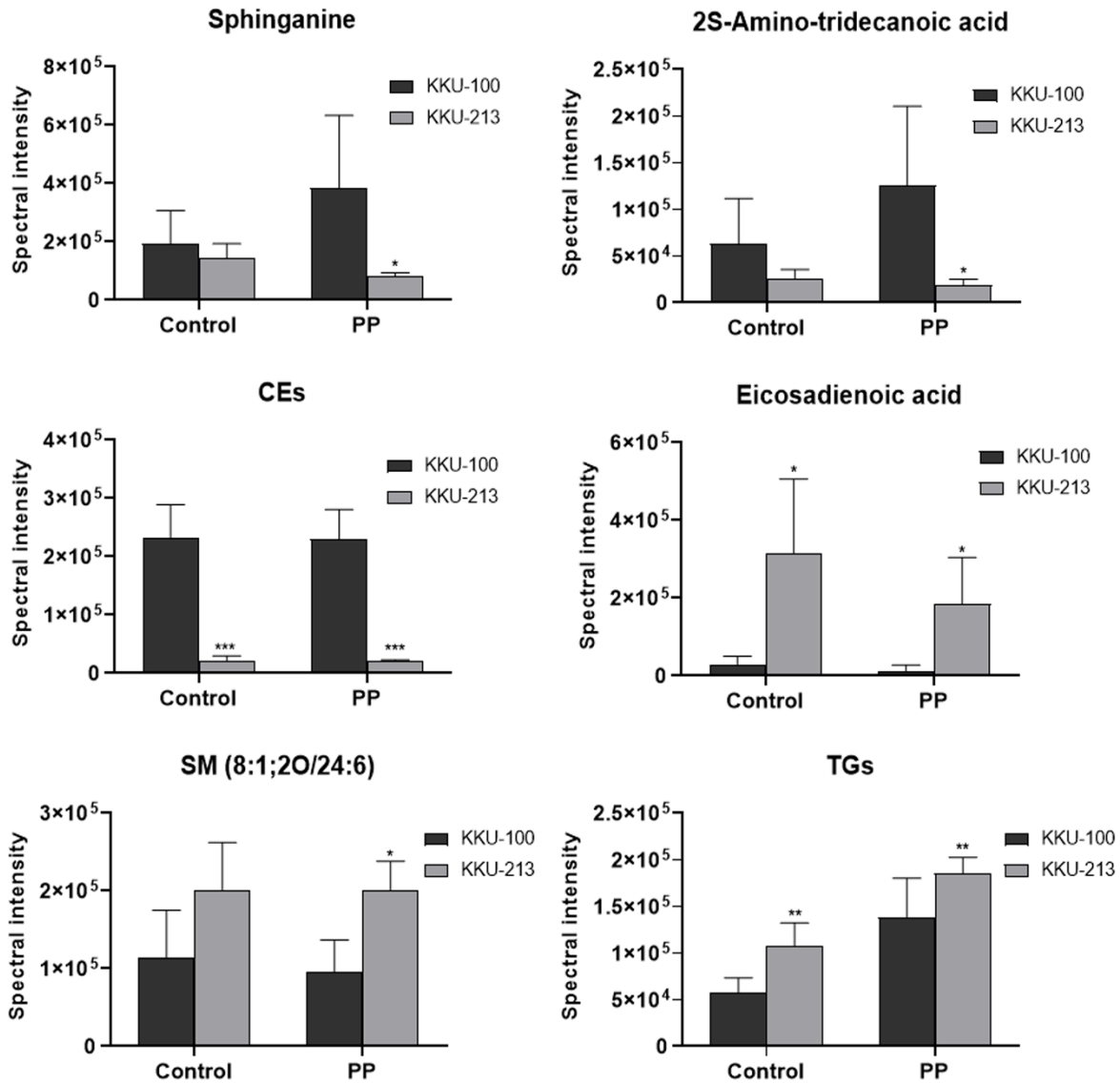
36 **Figure S1.** Multivariate analysis of the lipidomic profile of PP-treated CCA cells in negative and
 37 positive modes. (a,c) PCA ($R^2X= 0.883$, $Q^2=0.0843$ and $R^2X= 0.904$, $Q^2=0.788$, respectively)

38 (b,d) O-PLS-RE score plot ($R^2X= 0.757$, $Q^2Y=0.885$, CV-ANOVA $p= 0.014$ and $R^2X= 0.727$,
 39 $Q^2Y=0.693$, CV-ANOVA $p= 0.142$, respectively) of K KU-100 with PP-treated and control groups
 40 in negative and positive modes. (e,g) PCA ($R^2X= 0.862$, $Q^2=0.177$ and $R^2X= 0.899$, $Q^2=0.792$,
 41 respectively) and (f,h) O-PLS-RE score plot ($R^2X= 0.836$, $Q^2Y=0.438$, CV-ANOVA $p= 0.497$ and
 42 $R^2X= 0.801$, $Q^2Y=0.901$, CV-ANOVA $p= 0.010$, respectively) of K KU-213 with PP-treated and
 43 control groups in negative and positive modes, respectively. (n=5 for each group).



44

45 **Figure S2.** Multivariate analysis of the lipidomic profile signature of control and PP-treated CCA
 46 cells in negative and positive modes. (a, b) O-PLS-DA score plot of negative mode based on the
 47 lipid profile in the control and PP-treated groups of K KU-100 vs. K KU-213 cells ($R^2X= 0.855$,
 48 $Q^2Y=0.712$, CV-ANOVA $p= 0.124$ and $R^2X= 0.703$, $Q^2Y=0.636$, CV-ANOVA $p= 0.301$,
 49 respectively) (c, d) O-PLS-DA score plot of positive mode in control and PP-treated groups of
 50 K KU-100 vs. K KU-213 ($R^2X= 0.789$, $Q^2Y=0.916$, CV-ANOVA $p= 0.007$ and $R^2X= 0.832$,
 51 $Q^2Y=0.844$, CV-ANOVA $p= 0.030$, respectively).



52

53 **Figure S3.** Relative concentrations of lipid signatures in control and PP-treated groups of KKU-
 54 100 vs. KKU-213 cells including sphinganine, 2S-Amino-tridecanoic acid, cholesteryl esters
 55 (CEs), eicosadienoic acid, sphingomyelin; SM (8:1;2O/24:6) and triglycerides (TGs). Error bars
 56 represent the standard deviation (SD) of samples (n=5). The significant difference was determined
 57 using unpaired t-tests (*p < 0.05, **p < 0.01, ***p < 0.001, ns is no significant difference) compared
 58 to control group.

59 **Supplementary table S1.** The altered lipid species identification for K KU-100 and K KU-213 cells after treatment with PP in positive
 60 and negative modes.

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	VIP	Delta(ppm)	MS/MS fragment ion (m/z)
KKU-100								
1	S-Acetyldihydrolipoamide	[M-H] ⁻	248.95	1.09	0.80	1.11	0.87	-
2	Methylselenopyruvate	[M-H] ⁻	180.96	1.13	0.84	1.23	105	-
KKU-213								
3	Sphinganine	[M+H] ⁺	274.27	1.01	0.62	2.42	0.00	88.07; 321.25; 274.27
4	PI (20:4/0:0)	[M+NH4] ⁺	638.34	3.38	0.76	1.84	9.00	-
5	TG (18:0/22:5/22:7)	[M+NH4] ⁺	996.80	15.64	0.99	2.10	2.68	-
6	TG (16:0/22:4/22:7)	[M+NH4] ⁺	970.78	15.65	1.00	2.58	0.31	201.16; 623.50
7	TG (12:0/22:1/22:7)	[M+NH4] ⁺	920.77	15.67	0.99	3.07	0.18	201.16; 647.51
8	TG (16:1/18:2/20:4)	[M+NH4] ⁺	896.77	15.75	0.99	3.10	2.81	311.26; 597.49
9	TG (18:1/20:4/20:4)	[M+NH4] ⁺	946.78	15.76	0.99	4.52	1.03	203.18; 601.52
10	TG (12:0/16:0/22:4)	[M+NH4] ⁺	844.74	15.76	0.99	1.92	1.1	-

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	VIP	Delta(ppm)	MS/MS fragment ion (m/z)
11	TG (16:0/22:3/22:7)	[M+NH ₄] ⁺	972.80	15.79	0.99	2.66	0.12	265.25; 625.52
12	TG (12:0/18:0/22:5)	[M+H] ⁺	870.75	15.81	0.99	2.43	1.47	-
13	TG (14:0/22:1/22:7)	[M+H] ⁺	953.75	15.91	0.98	1.95	3.24	-
14	TG (12:0/22:0/22:7)	[M+H] ⁺	922.78	15.92	1.00	5.52	2.44	-
15	TG (12:0/16:0/22:3)	[M+NH ₄] ⁺	846.75	16.02	0.99	4.18	1.96	-
16	TG (12:0/14:0/22:2)	[M+NH ₄] ⁺	820.74	16.03	0.99	2.75	0.98	-
17	TG (13:0/22:0/22:7)	[M+NH ₄] ⁺	936.80	16.03	0.98	1.71	2.87	-
18	TG (12:0/21:0/22:6)	[M+H] ⁺	910.78	16.05	0.99	2.26	0.55	-
19	TG (12:0/20:0/22:5)	[M+N _{H4}] ⁺	898.78	16.05	0.99	4.95	1.59	-
20	TG (12:0/18:0/22:4)	[M+NH ₄] ⁺	872.77	16.06	0.99	5.09	1.65	261.22; 575.51
21	TG (16:0/20:3/20:4)	[M+NH ₄] ⁺	922.78	16.08	0.99	5.89	2.44	313.26; 623.51; 905.76
22	TG (12:0/19:0/22:4)	[M+NH ₄] ⁺	886.78	16.17	0.99	2.69	2.75	-
23	TG (14:0/22:0/22:7)	[M+Na] ⁺	955.77	16.18	0.97	1.84	0.43	-
24	TG (18:1/18:1/22:5)	[M+NH ₄] ⁺	950.81	16.18	0.98	4.95	3.73	339.29; 651.54; 933.794

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	VIP	Delta(ppm)	MS/MS fragment ion (m/z)
25	TG (12:0/17:0/22:3)	[M+NH ₄] ⁺	860.77	16.18	0.99	2.89	2.99	-
26	TG (16:0/22:1/22:7)	[M+H] ⁺	976.83	16.19	0.99	1.83	3.29	-
27	TG (12:0/15:0/22:2)	[M+H] ⁺	834.75	16.19	0.99	1.80	1.65	-
28	TG (12:0/22:0/22:6)	[M+H] ⁺	924.80	16.2	0.99	6.97	2.81	-
29	TG (18:1/18:1/18:2)	[M+NH ₄] ⁺	900.80	16.33	0.98	6.63	3.48	263.24; 601.52
30	TG (16:0/18:1/18:2)	[M+NH ₄] ⁺	874.78	16.33	0.98	7.99	1.77	263.24; 575.51
31	TG (12:0/14:0/22:1)	[M+NH ₄] ⁺	822.75	16.33	0.70	2.03	1.35	-
32	TG (12:0/18:0/22:3)	[M+Na] ⁺	879.74	16.34	0.98	2.26	1.46	-
33	TG (12:0/20:0/22:4)	[M+Na] ⁺	905.75	16.34	0.95	1.83	1.9	-
34	TG (16:0/16:1/18:1)	[M+NH ₄] ⁺	848.77	16.34	0.97	4.80	1.65	311.26; 549.49
35	TG (13:0/22:0/22:6)	[M+Na] ⁺	938.81	16.34	0.99	1.73	4.03	-
36	TG (12:0/22:0/22:5)	[M+H] ⁺	926.81	16.4	0.99	3.50	0.67	289.25; 577.52
37	TG (12:0/19:0/22:3)	[M+NH ₄] ⁺	888.80	16.45	0.96	2.68	2.62	313.28; 589.52
38	TG (12:0/17:0/22:2)	[M+NH ₄] ⁺	862.78	16.46	0.93	2.95	1.35	-

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	VIP	Delta(ppm)	MS/MS fragment ion (m/z)
39	TG (12:0/15:0/22:1)	[M+NH ₄] ⁺	836.77	16.48	0.90	1.89	0.73	-
40	TG (14:0/22:0/22:6)	[M+NH ₄] ⁺	952.83	16.5	0.99	4.07	2.32	-
41	TG (12:0/20:0/22:3)	[M+H] ⁺	902.81	16.59	0.84	4.30	1.77	-
42	TG (12:0/18:0/22:2)	[M+NH ₄] ⁺	876.80	16.6	0.69	4.83	0.85	-
43	TG (12:0/22:0/22:4)	[M+H] ⁺	928.83	16.61	0.96	2.59	3.35	265.25; 605.55
44	TG (14:0/22:0/22:5)	[M+NH ₄] ⁺	954.84	16.7	0.99	2.06	4.15	-
45	TG (12:0/19:0/22:2)	[M+H] ⁺	890.81	16.74	0.67	1.91	1.65	95.09; 577.52

61 PI, phosphatidylinositol; TG, triglyceride; m/z, observed mass-to-charge ratio; RT, retention time (min).

62

63

64

65

66

67

68

69 **Supplementary table S2.** The lipid signature identification of K KU-100 and K KU-213 cells.

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	Delta (ppm)	MS/MS fragment ion (m/z)
KKU-100							
1	Sphinganine	[M+H] ⁺	274.27	1.01	0.52	0.00	88.07; 321.25; 274.27
2	2S-Amino-tridecanoic acid	[M+H] ⁺	230.25	1.03	0.66	0.04	228.27
3	CE (20:5)	[M+NH4] ⁺	688.60	16.00	0.92	0.92	369.35
4	CE (22:6)	[M+NH4] ⁺	714.62	16.12	0.96	0.61	161.13; 369.35
5	SE (28:2/17:2)	[M+H] ⁺	664.60	16.20	0.96	3.05	-
6	CE (20:4)	[M+NH4] ⁺	690.62	16.27	0.96	1.40	109.10; 369.35
7	CE (22:5)	[M+NH4] ⁺	716.63	16.30	0.97	2.02	233.23; 369.35
8	SE (28:2/15:0)	[M+H] ⁺	640.60	16.49	0.95	1.59	-
9	CE (18:2)	[M+NH4] ⁺	666.62	16.51	0.95	1.03	243.21; 369.35
10	CE (20:3)	[M+NH4] ⁺	692.63	16.54	0.96	1.53	179.18; 369.35
11	SE (28:2/21:3)	[M+NH4] ⁺	718.65	16.60	0.96	0.00	179.18; 718.58
12	SE (29:1/22:5)	[M+H] ⁺	744.66	16.64	0.97	0.55	-

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	Delta (ppm)	MS/MS fragment ion (m/z)
13	SE (28:2/19:1)	[M+H] ⁺	694.64	16.84	0.97	0.55	153.09
14	CE (18:1)	[M+NH4] ⁺	668.63	16.85	0.97	0.18	135.12; 369.35
15	SE (28:2/21:2)	[M+H] ⁺	720.66	16.88	0.95	0.19	-
16	CE (20:1)	[M+NH4] ⁺	696.66	17.15	0.94	0.55	153.09; 369.53
17	SE (28:2/21:1)	[M+H] ⁺	722.68	17.16	0.93	0.31	-
KKU-213							
18	Eicosadienoic acid	[M+H] ⁺	387.19	1.01	0.67	1.00	79.05; 93.07; 105.07; 119.05
19	SM (8:1;2O/24:6)	[M+H] ⁺	663.45	9.60	0.84	2.68	227.20; 485.27
20	TG (18:2/18:2/18:2)	[M+NH4] ⁺	896.77	15.75	0.96	0.61	261.22; 599.51
21	TG (18:1/20:4/20:4)	[M+NH4] ⁺	946.78	15.76	0.84	1.58	203.18; 601.52
22	TG (8:0/12:0/38:6)	[M+NH4] ⁺	952.83	16.50	0.62	0.80	339.29; 651.54
23	TG (18:0/18:1/18:1)	[M+NH4] ⁺	904.83	16.88	0.56	0.48	135.12; 605.55; 904.84

70 CE, cholesteryl ester; SE, sterol ester; SM, sphingomyelin, TG, triglyceride; m/z, observed mass-to-charge ratio; RT, retention time
71 (min)

Supplementary table S3. The candidate lipid species identification of KKU-100 and KKU-213 cells associated with mitochondrial membrane potential after treatment with PP.

No.	Lipid molecular species	Ion species	m/z	RT	p(corr)	VIP (ATP)	VIP (TMRE)	Delta (ppm)	MS/MS fragment ion (m/z)
1	TG (16:0/18:1/18:1)	[M+NH4] ⁺	876.80	16.60	0.91	9.54	9.54	1.59	265.25; 577.52; 841.78
2	TG (16:0/18:1/18:2)	[M+NH4] ⁺	874.78	16.33	0.94	8.78	8.81	2.07	263.24; 575.51
3	TG (18:0/18:3/20:3)	[M+NH4] ⁺	924.80	16.20	0.92	8.57	8.56	1.71	313.26; 578.52
4	TG (16:0/16:1/18:1)	[M+NH4] ⁺	848.77	16.34	0.93	6.80	6.82	2.32	311.26; 549.49
5	TG (18:1/18:1/18:2)	[M+NH4] ⁺	900.80	16.33	0.92	6.57	6.57	1.53	263.24; 601.52
6	TG (18:1/18:1/18:1)	[M+NH4] ⁺	902.81	16.59	0.93	6.30	6.32	1.28	265.25; 603.54; 902.83
7	TG (16:0/20:3/20:4)	[M+NH4] ⁺	922.78	16.08	0.92	6.11	6.11	0.36	313.26; 623.51
8	TG (12:0/16:0/22:3)	[M+NH4] ⁺	846.75	16.02	0.92	5.69	5.71	0.55	311.26; 547.47
9	TG (8:0/8:0/36:4)	[M+NH4] ⁺	872.77	16.06	0.91	5.69	5.69	0.49	261.22; 575.05
10	TG (18:1/18:1/22:5)	[M+NH4] ⁺	950.81	16.18	0.94	5.21	5.22	0.92	339.29; 651.54; 933.79

TG, triglyceride; m/z, observed mass-to-charge ratio; RT, retention time (min)