Supporting Information for:

Metabolomics study on the cytotoxicity of graphene

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Experimental section

Materials and reagents

Acetonitrile, methanol and formic acid are chromatographic grade and purchased from Sigma (Sigma-Aldrich). Graphite powder (purity>99%) was purchased from Qingdao Tianhe Graphite Co. Ltd. (Qingdao, China). HepG2 cells were provided by Harbin Medical University (Harbin, China). Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin, and streptomycin were obtained from Invitrogen. Fetal bovine serum (FBS) was purchased from Sigma (Sigma-Aldrich). Culture dish, culture flask, and cell scraper were all purchased from Corning (Corning, USA). Other reagents were analytical grade.

Graphene and sample preparation

Water-soluble graphene nanosheets used in this case were prepared using our previously reported method.[1] HepG2 cells were gathered at fifth generation, and then planted into the culture dish with diameter 8 cm at the density of 1.5×10^6. After the HepG2 cells were added in the dishes, they were kept in the incubator at 37 °C for overnight. Then the different concentrations water-soluble graphene solutions (0, 0.025, 0.4, 1mg/mL) were added to the control, low, middle, and high group, respectively. At the special interval of time 24 h, 48 h, and 72 h respectively, we employed the improved method from Matthew A. Lorenz et al.[2] to pool the endogenous metabolites of HepG2 cell. The first, we used 10 mL liquid nitrogen to quench the protease for stopping the enzyme activity. Second, the HepG2 cells were swiftly pooled with cell scraper. At last, we adopted the chromatograph methanol as

the precipitant to get rid of the protein, and then centrifuged with 10000 rpm for 15 min, at 4 °C. The methanol aqueous were transferred another tube and dried under nitrogen gas. Lastly, the sample tube was added methanol-ultrapure water (2:1, V/V) and vortex ten min for analysis.

Chromatography and mass spectrometry condition

The improved chromatography condition was carried out following previously reported methods.[2,3,4] Waters ACQUITY UPLC system, binary solvent delivery system and auto-sampler (Waters, USA) were employed. Every liquid sample, 5 μL solution was injected into the ACQUITY BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, USA) with column temperature maintained at 35 °C, and the eluted flow rate was set 0.4 mL/min. The eluent was directed into the mass spectrometer without split. We carried out the optimal linear gradient system with the mobile phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile). Gradient as follow: 0-2 min, 99%-70% A; 2-8 min 70%-1% A; 8-10 min, 1%-1% A;10-10.1 min,1-99% A, 10.1-12.5 min, 99%-99%, A.

The improved mass condition[3, 4] was operated. The column eluent was directly introduced into mass spectrometer to analysis. MS data were acquired in the positive mode (ESI^+) from 0 to 12.5 min in full scan auto mode and scan rate was kept 1.03 spectra/s with the range of scan from 50 to 1000 m/z. Optimal analysis parameters as follow: In positive mode the capillary voltage was set 3.2 KV, sample cone voltage was of 32 V, the flow of desolvation gas was maintained 600 L/h and kept its temperature 330 °C, the cone gas flow was of 30 L/h and the source temperature was

kept 115°C. It has been calibrated before usage for guarantee the accuracy and the stability of the equipment.

Data processing

All mass data acquired from the UPLC-MS was managed by virtue of Ezinfo 2.0 software (Waters, USA) for unsupervised analysis and supervised analysis, namely, PCA and PLS-DA. Firstly, in PCA and PLS-DA, the data sets were mean-centered and employed Pareto as the scale type for X-variables. We therefore got the clustering groups, that is, finding the discrepancy between treated groups and control group. Secondly, the contribution rate of the metabolite for separation the control group from the treated group in loading plot and VIP plot was operated. The metabolites scattering away from the original point in loading plot while screening a VIP value (VIP>1) in VIP score plot were seen as the pronounced markers. Thirdly, univariate statistical analysis was performed by GraphPad Prism5.0 for further identification the potential markers, including box figure analysis and ANOVA, and *p*-value was set as 0.05 for statistical significance.

Identified biomarker and pathway

By the means of the loading plot, VIP>1, and with significant difference in the univariate analysis, the potential markers were acquired. To begin with, the precursor ion of metabolite, retention time, m/z, and the fragmentation ion pattern of the interesting metabolite were employed UPLC-Q-TOF-MS/MS to acquire. What's more, some rules were adopted from previously reported methods[5, 6] to compare with the data from database KEGG, http://www.genome.jp/kegg/kegg1.html; METLIN,

http: //metlin.scripps.edu/; HMDB, http://www.hmdb.ca/; and Mass Bank, http://www.massbank.jp/, etc. to deciphered the clearly structure. Finally, all the unequivocal metabolites were imported into the above mentioned database to construct, interpret, and draw the pathway.

References:

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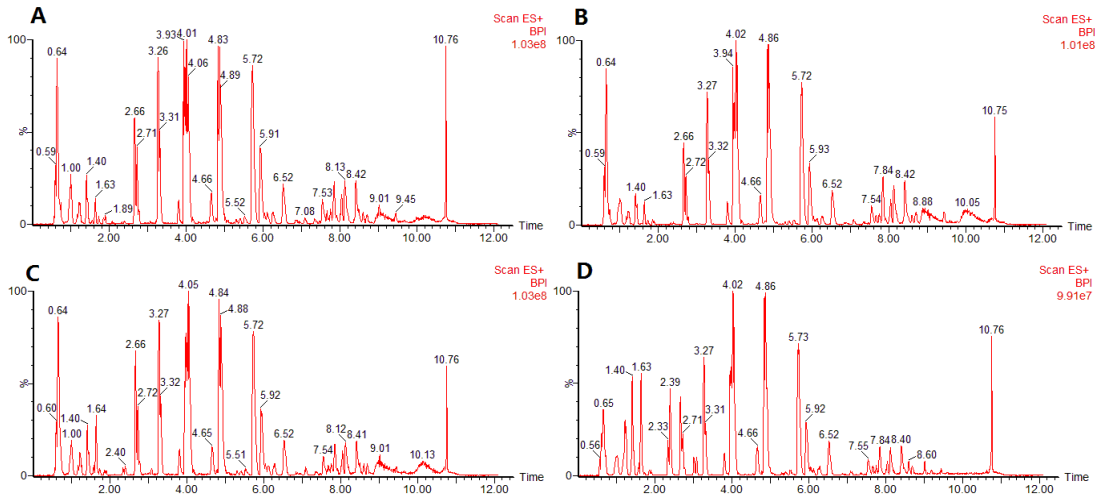

Fig. S1. The BPI chromatography of the metabolites of HepG2 cell in positive mode: A. control group, B. low dose group, C. middle dose group, D. high dose group.

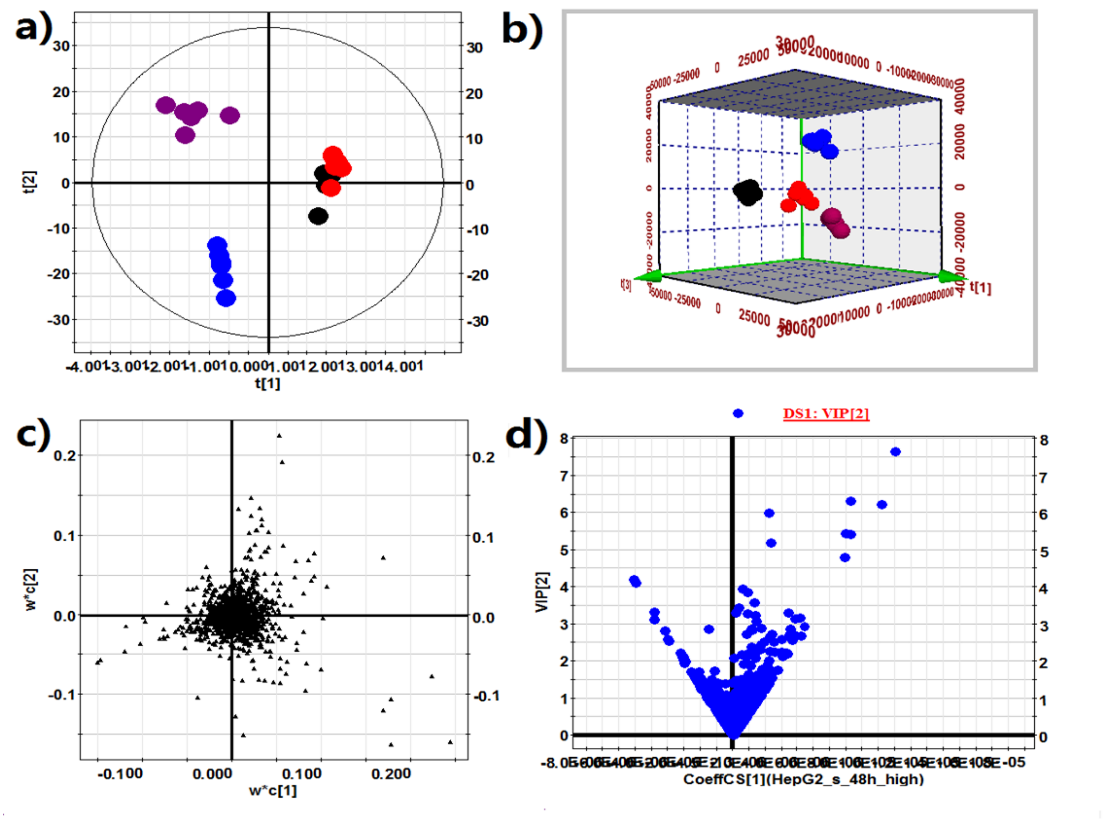

Fig. S2. Multivariate analysis of UPLC-Q-TOF-MS data for 48 h graphene- treated groups and control group. (a) PCA score plot. (b) 3D PLS-DA score plot. (c) Loading plot of PLS-DA. (d).VIP score plot from PLS-DA. Control group (black, graphene: 0 mg/mL), low dose group (red, graphene: 0.025 mg/mL), middle dose group (blue, graphene: 0.4 mg/mL), and high dose group (violet, graphene: 1 mg/mL).

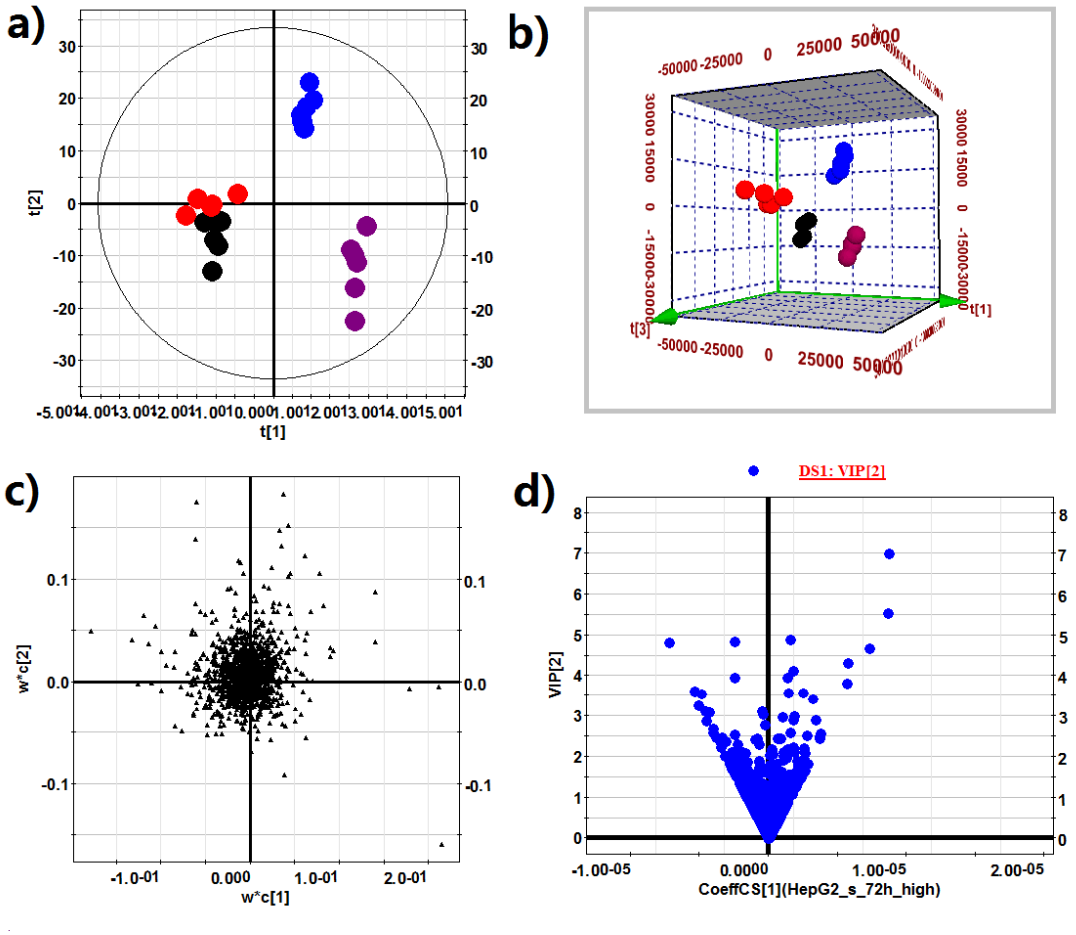

Fig. S3. Multivariate analysis of UPLC-Q-TOF-MS data for 72 h graphene-treated groups and control group. (a) PCA score plot. (b) 3D PLS-DA score plot. (c) Loading plot of PLS-DA. (d).VIP score plot from PLS-DA. Control group (black, graphene: 0 mg/mL), low dose group (red, graphene: 0.025 mg/mL), middle dose group (blue, graphene: 0.4 mg/mL), and high dose group (violet, graphene: 1 mg/mL).

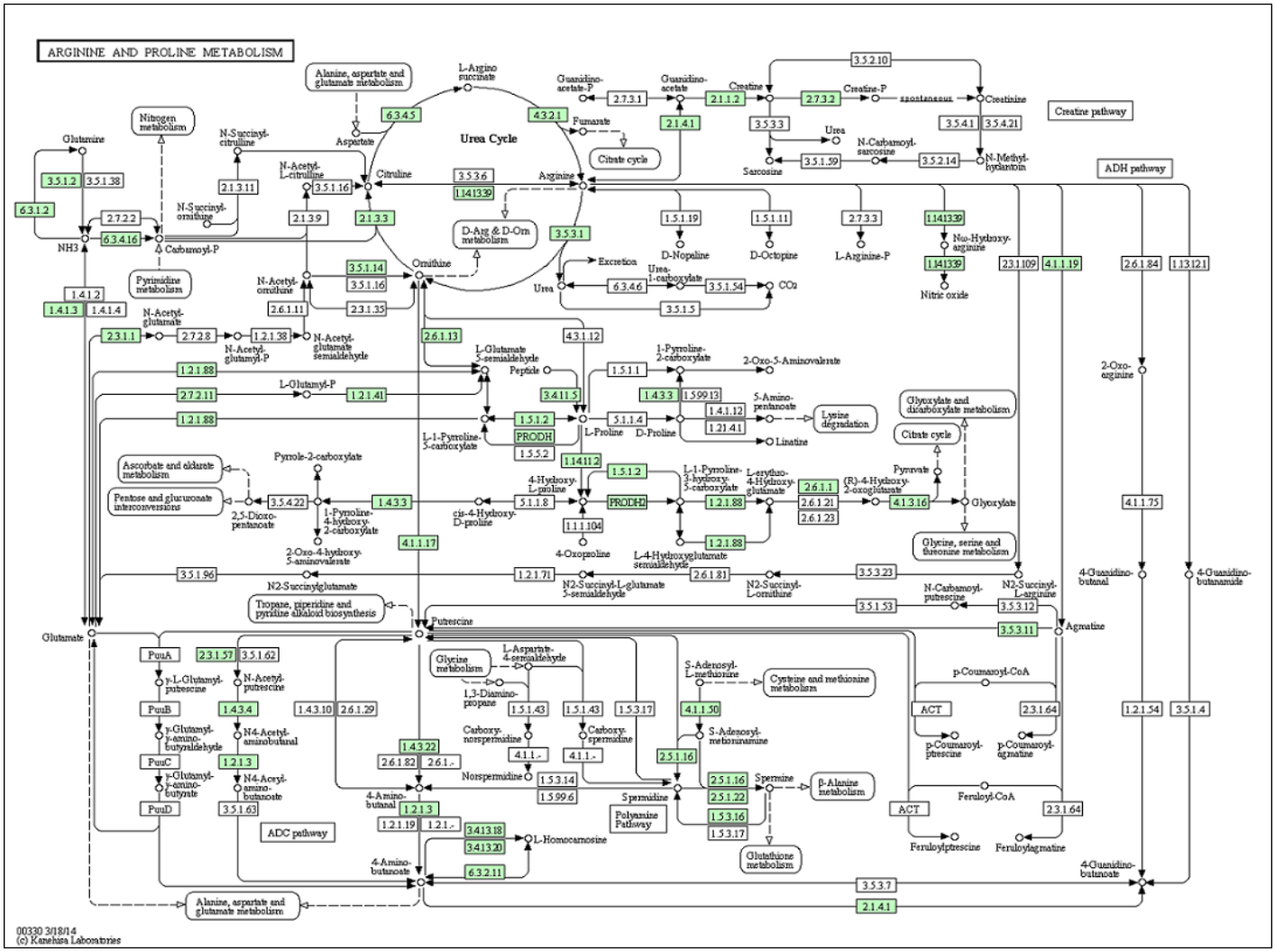

Fig. S4. Construction of the arginine and proline metabolism pathways in graphene-treated HepG2 based on KEGG database (http: //www. genome.jp/kegg/). The green rectangles denote enzymatic activities

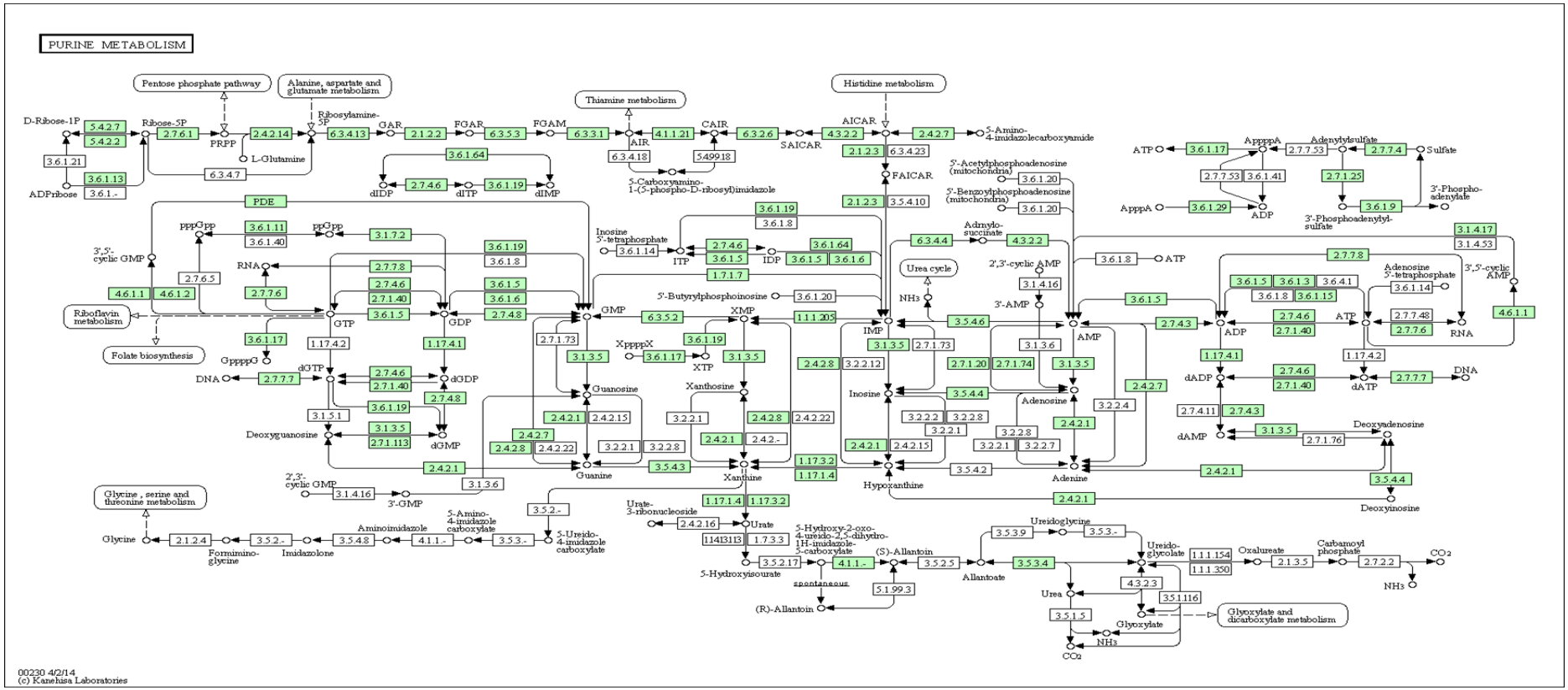

Fig. S5. Construction of the purine metabolism pathways in graphene-treated HepG2 based on KEGG database (http: //www. genome.jp/kegg/). The green rectangles denote enzymatic activities.

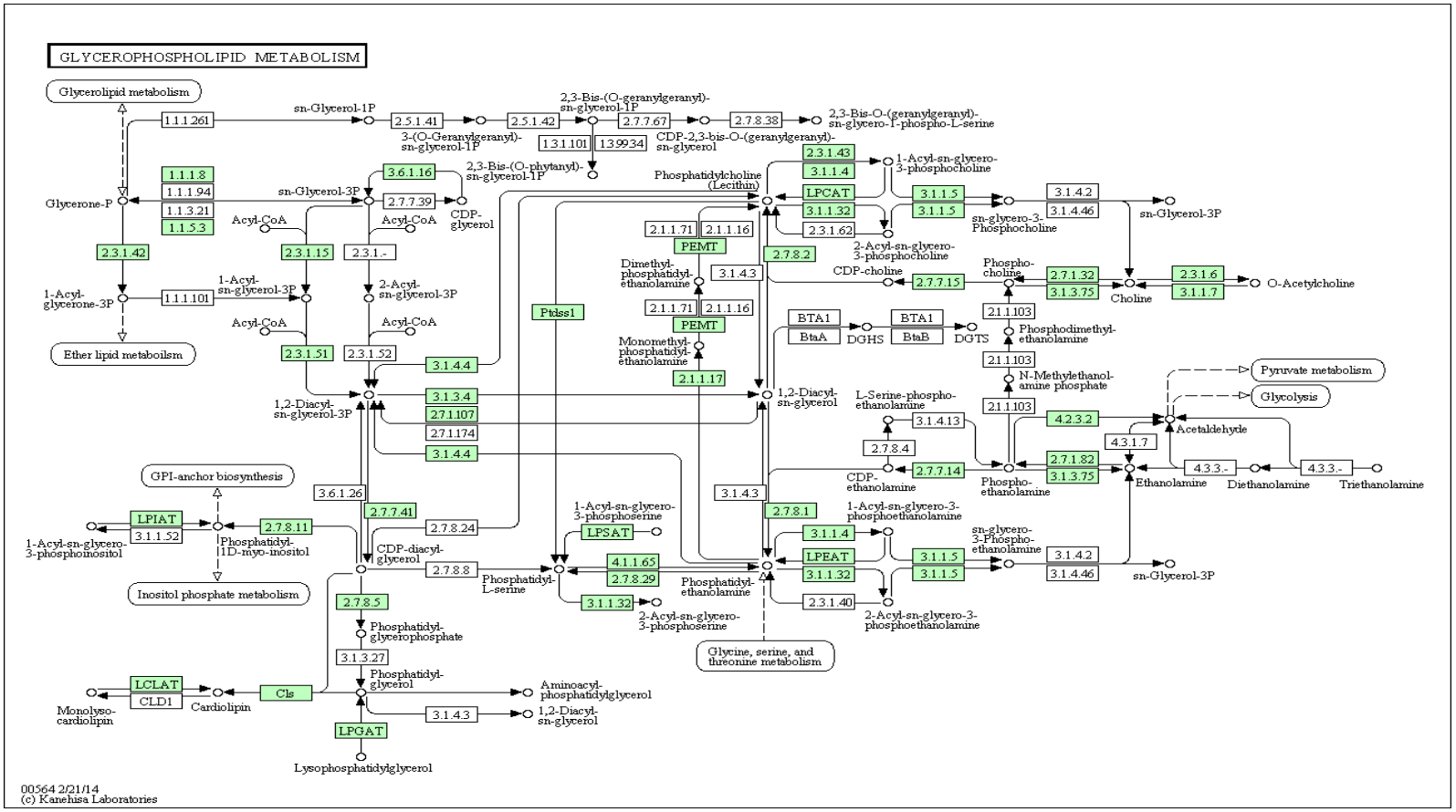

Fig. S6. Construction of the glycophospholipid metabolism pathways in graphene-treated HepG2 based on KEGG database (http: //www. genome.jp/kegg/). The green rectangles denote enzymatic activities.