Raman-deutrium isotope probing to evaluate the cytotoxicity of anticancer drug to cancer cells

Xia Huang[1], Chengjian Li[2], Guilan Jin[3], Lihao Zhang[1], Xiaofei Yi[3], Yun Wang[4], Guangxin Liu[1], Xiangtai Zheng[2], Yizhi Song, Wei E. Huang[5]*and Liang Zhao[2]*

[1]Suzhou Institute of BiomedicalEngineering and Technology, Chinese Academy of Sciences,Keling Road, Suzhou, Jiangsu Province, 215163, China

[2]Luodian Clinical Drug Research Center, Institute for Translational Medicine Research, Shanghai University, Shanghai, China.201908

[3]Shanghai D-band Medical Instrument Co. Ltd, Huyi Highway, Jiading District, Shanghai, 201800, China

[4]Shanghai Hesen Biotechnology Co. Ltd, Huyi Highway, Jiading District, Shanghai, 201800, China

[5]Department of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, UK

*Corresponding author.

* Wei E. Huang, email: wei.huang@eng.ox.ac.uk Tel: +44-01865-283786.

* Liang Zhao, e-mail: zhaoliangphar@163.com Tel: +86-021-66861212-6303.

Abstract

The evaluation of anticancer drug to tumour cells is important to clinical practice. Raman microspectroscopy-deutrium isotope probing (Raman-DIP) has been applied to study the response of breast cancer cell line (MCF7 cells) to an anticancer drug (GSK233470) *in vitro*. The cytotoxicity analysis of Raman-DIP was consistent to conventional cell counting kit 8 (CCK8) assay, and both methods indicated that 10 μM of GSK233470 was the cytotoxic concentration for regeneration inhibition to cancer cell line MCF7. Single-Cell Raman Spectra were also used for sensitivity testing of targeted anti-cancer drugs. The variation of the bio-macromolecules reflected by Raman signal in the fingerprint range were closely related to the GSK233470 concentration. In brief, we propose that Raman DIP method can be used to monitor the inhibition of the GSK233470 to the MCF7 cell proliferation with high accuracy as CCK8 assay. The combination of Raman DIP results and fingerprint range information could effectively improve the evaluation, which could be a supplement for the existing anti-cancer drug sensibility assay.

Keywords: Raman spectroscopy; Breast cancer; Deuterium isotope probing; Anticancer drug screening

Introduction

Breast cancer is one of the most common cancer among women world widely and chemotherapy is a main procedure during the cancer treatment [1, 2]. Recent development in the targeted anticancer drugs helped to improve the treatment and ease the adverse drug reactions. However, the therapeutic performance varies greatly among individuals regarding to the patient's physical conditions and the drug concentration. In vitro experiments, the chemotherapy outcome can be influenced by several elements, for instance the cell populations, the experimental condition of the cell regeneration, the drug concentration. Therefore, cytotoxicity test became a useful way to evaluate the drug performance in vitro. Among all the cytotoxicity test methods, the MTT assay is commonly used to assess cell viability by monitoring its mitochondrial activities [3]. However, targeted anticancer drugs have fewer inhibitory effects on tumour proliferation compared to cytotoxic anticancer drugs, so conventional cytotoxicity testing is unsuitable for evaluating targeted anticancer drugs. Currently, sensitivity to targeted drugs is usually evaluated by molecular biological methods, such as gene mutation analysis, gene polymorphism analysis, and gene expression detection.

Raman spectroscopy is a label-free and non-destructive approach that provides intrinsic molecular profile of a single cell. Single-cell Raman Spectra (SCRS) is widely used in biomedical field, for instance, discriminating cancer cells from the normal ones [4], tracking the drug biding sites, analyse the components in cancer cells, monitoring anticancer drug treatment [5], et al. The combination of stable isotope probing and Raman spectroscopy can reveal more information on cell metabolism [6]. Among all the isotopes, deuterium is the one with universality as H_2O is widely involved in cell metabolism, such as the synthesis of lipids, fatty acids, proteins, and nucleic acids. The deuterium isotope of hydrogen (D_2O) can substitute hydrogen in water and participate in cell metabolism. Thus, using Raman-DIP (Deuterium Isotope Probing) method, instead of forming C-H bands at around 2800-

3000 cm^{-1}, the C-D bonds around 2040-2300 cm^{-1} was formed during the metabolic activities [7, 8]. According to this, Song et al. who used heavy water (D_2O) to label the environmental bacteria and studied the antimicrobial resistance of the bacteria [9]. We hypothesis that after exposing to the targeted drugs, cells are supposed to become less active due to the inhibiting effect from the drugs. By adding heavy water into the incubation buffer, the intensity ratio between the C-D band and the C-H band can be monitored, which reveals the metabolism rate of the cancer cells and the inhibiting effects of the targeted drugs on the cancer cells at a single-cell level. In addition, the fingerprint range of the Raman spectra from 400 cm^{-1} to 1800 cm^{-1} can provide much information like the cell composition, structural conformation and can also provide specific diagnostic information about backbone structures of protein, nucleic acid in cells [10]. Thus, by analysing the fingerprint range of the cancer cells, the inhibiting effects of the targeted drugs could also be evaluated.

In breast cancer treatment, 3-phosphoinositide dependent protein kinase-1 (PDK-1) was used as targets for estimating molecularly targeted drug effects. PDK-1 functions down stream of phosphatidylinositol 3-kinase (PI3K) and activates members of the Bcl-2 family of protein kinases that is known to play a key role in physiological processes associated with cell metabolism, growth, proliferation and survival [11, 12]. Breast cancer express high level of PDK-1 [13], so the inhibitors of PDK-1 signalling has therapeutic potential in suppressing the growth of breast cancer. GSK233470 is one of PDK-1 inhibitors, which can decrease the phosphorylation of PDK1 and activate protein kinase B (Akt) [14]. This could lead to the upregulation of Cytochrome C and the downregulation of Bcl-2 triggering cell death [15].

In this study, we used Raman-DIP method to study the response of the human breast adenocarcinoma cells (the MCF7 cells) to the targeted anticancer drug GSK233470 *in vitro*. The result from the Raman-DIP method was compared with the that from the widely used CCK8 assay, and the stability and reproducibility of the Raman-DIP method was verified as

an anti-cancer drug sensitivity assay. The parametric correlation coefficient of drug responses derived from CCK8 and Raman-DIP results were calculated with Pearson's correlation coefficient. Pharmacologically, the dose-response relationship describes the change in the effect on cells caused by different levels of exposure (doses) to a drug for a certain exposure time, and dose-response curves are often fit to a classical Hill equation. Here, we fit the relationship between drug dose and the cell response (RSI). The results indicated that our experimental data identified with the dose-response relationship. Furthermore, we showed that Raman-DIP method can be used for sensitivity testing of targeted anti-cancer drugs. In brief, we propose a new method of quantifying heterogeneous characteristics of cancer cells by treating cells with drugs. This method supplements the existing drug sensibility test method.

Materials and Methods

Solution preparations

MCF-7 cells were routinely cultured in culturing solution with 5% CO_2 at 37°C for 24 hours. The culturing solution consists of DMEM (Gibco Company, USA) ,10% fetal bovine serum (Gibco Company, USA), penicillin and streptomycin (Gibco Company, USA).

GSK233470 (FINE Scientific Ltd., USA) was diluted in DMEM to make a 10 mg mL^{-1} stock solution.

D2O (Huaxia Reagent Co., Ltd., China) was filtered by membrane with 0.22 μm pores (Merck Millipore Ltd., Germany) before use.

CCK8 assay for cytoxicity test

MCF7 cells at a concentration of 10 thousand per well, were cultured in culturing solution with 5% CO_2 at 37°C for 24 hours using a 96-well plate. Cells were then treated with GSK233470 solution at 8 concentrations of 0 μM, 0.5 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, 20 μM for 48 hours. To assess the cytoxicity of GSK233470, the IC50 value were obtained from a Cell Counting Kit-8 (CCK-8) assay (GLPBIO, Montclair, CA, USA), which refers to a 50% inhibiting concentration of cell growth.

D_2O treatment for Raman test

MCF7 Cells at a concentration of 10 million cells per well, were cultured in culturing solution with 5% CO_2 at 37°C for 24 hours using 6-well plates. Cells were then treated with GSK233470 solution at different concentrations of 0 μM, 0.5 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, 20 μM for 24 hours, followed by incubating with D_2O (30% v/v) for another 48 hours. After the treatments, cells were washed 3 times using PBS buffer (PBS, *Gibco* Company, USA). Finally, 2 μl of cell suspension was deposited on a piece of aluminum-coated slide (Shanghai D-Band Medical instrument co., Ltd) for each GSK233470 concentration and left dry for Raman measurement.

Raman measurement and data analysis for cytoxicity test

The Raman properties of samples were measured on a WITec alpha300 Raman microscope (WITec GmbH, Germany). The microscope is equipped with 2 sets of light sources: a 532 nm laser for Raman measurement; and a LED white light for wide field images. The excitation light is focused by a 100 × objective (Epiplan-Neofluar, NA = 0.9, Zeiss) and the Raman emission is collected from the same focal spot on the sample. The laser power on sample was around 8 mW and a grating with 600 lines mm^{-1} was used to spread the Raman emission for wavelength recording. For each sample, 150 Raman spectra were acquired with the spectrum ranging from 400 to 3400cm^{-1} and with the acquisition time set to 3 seconds per spectrum.

Raman spectra were analysed by Project FIVE 5.1 (WITec, Germany) and Labspec 6 (HORIBA, Japan), the routine data analysis including cosmic ray removal, background subtraction and normalization. The normalization was performed referring to the area of C-H band between 2800 cm^{-1} and 3100 cm^{-1}. The C-D/C-H band intensity ratios were used to assess the metabolic activity of the cells. The C-D/C-H band intensity ratios were calculated by their integrated band area as from 2040 cm^{-1} to 2300 cm^{-1} for C-D band and from 2800 cm^{-1} to 3100 cm^{-1} for C-H band. The Raman fingerprint range from 400 cm^{-1} to 1800 cm^{-1} was used to study the effect of GSK233470 on cells. The 150 spectra from each sample was averaged for comparation.

Results

PDK1 inhibitor cytotoxicity evaluated by CCK8 assay

MCF7 cells were cultured in culturing solution for 24 hours and then treated with GSK233470 at 8 different concentrations for 48 hours. The CCK8 assay kit was applied to evaluate the PDK1 inhibitor cytotoxicity according to the cellular survival rate, shown as cell visibility (Fig. 1). The IC_{50} (half maximal inhibitory concentration) was used to define the cytotoxicity of the drug. The calculation result from data Fig. 1 shows that the IC_{50} concentration of the GSK233470 was around 10 μM. As can be seen, most cells were still actively alive when GSK233470 concentration was below 5 μM, where the cell visibility varied from 93.9% to 63.7% when the GSK233470 concentration was between 0.5 μM and 5 μM. With GSK233470 concentration rose up to 10 μM, the cell visibility dropped to 50.9%, which matched the calculated GSK233470 cytotoxicity value (the IC_{50} concentration) for the cultured MCF7 cells. The cell visibility dropped to 16.3% and 7.5% for GSK233470 concentration at 15 μM and 20 μM, respectively.

Cell metabolism level and PDK1 inhibitor cytotoxicity evaluated by Raman DIP

MCF7 cells were cultured in culturing solution for 24 hours and then treated with GSK233470 at 8 different concentrations for 24 hours, followed by incubating with D_2O (30% v/v) for another 48 hours. After the treatments, the MCF7 cells went through digestive procedure and deposited on a chip for image. White light images were taken to show the morphological appearance of the MCF7 cells (Fig. 2). Regarding to positive control (cells, D_2O, no GSK233470) and negative control (cells, no D_2O, no GSK233470), cells showed a comparative population and reasonable shape when the GSK233470 concentration was below 10 μM. When GSK233470 concentration rose up to 10 μM, cells began to appear the cell atrophy on their shape and population. The GSK233470-induced cancer cell death that we observed showed the typical morphological features of oncosis, including the fact that these cells became bigger than normal living cells, and cellular volume obviously increased with the drug concentration.

Raman spectra of the MCF7 cells were taken after being treated with GSK233470 and incubated in D_2O. The C-D/(C-H+C-D) ratio was used to reflect the level of the cell metabolism and to evaluate the PDK1 inhibitor cytotoxicity. The C-D/(C-H+C-D) band intensity ratios were calculated by their integrated band area, as from 2040 cm^{-1} to 2300 cm^{-1} for the C-D band and from 2800 cm^{-1} to 3100 cm^{-1} for the C-H band. As can be seen in Fig. 3, the C-D band intensity dropped when the GSK233470 concentration rose. For the positive control (no GSK233470, 30% D_2O), the C-D/(C-H+C-D) ratio was 3.13%. The ratio gradually dropped to 1.37% when the GSK233470 concentration rose to 5 μM. A sharp decrease from 1.37% to 0.53% was seen when the GSK233470 concentration increased from 5 μM to 10 μM, which indicated that 10 μM of GSK233470 could effectively depress the metabolism of the MCF7 cells. The C-D/(C-H+C-D) ratio dropped to 0.14% and 0.15% when

the GSK233470 concentration were at 15 μM to 20 μM, respectively. For the negative control (no GSK233470, no D_2O), the C-D/(C-H+C-D) ratio was 0.15%.

Compare inhibition rates from Raman DIP result and CCK8 assay result

To verify the feasibility of Raman DIP method we compared it with CCK8 assay. The inhibition rates of GSK233470 to MCF7 cells were calculated based on Raman DIP results and CCK8 assay results, separately. The correlation of the inhibition rates based on the two methods was shown in Fig. 4. As can be seen, the inhibition rates from Raman DIP method were highly correlated with those from the CCK8 assay and the correlation coefficient value (R^2) was 0.88. Such a high correlation indicated that the Raman DIP method can be used to evaluate the GSK233470 inhibition just as the CCK8 assay and that the CCK8 assay and Raman DIP methods fitted the dose-response curves well.

Result of the fingerprint range analysis

In order to evaluate the feasibility of Raman spectrum applied in the targeted drug sensitivity assay, fingerprint range analysis was applied to MCF7 cells. Raman spectrum of bio-sample has an intrinsic fingerprint range between 400-1800 cm^{-1}. Raman bands in this range contain rich information about the intracellular proteins, nucleic acids, lipids, and other biological macromolecules. Thus, this fingerprint range reflects the physiological and biochemical status of the bio-sample. Here, we recorded the fingerprint range Raman signal of the MCF7 cells with and without GSK233470 treatment to study the drug effects on the cells through intracellular macromolecules. For each sample 150 Raman spectra were recorded, and the averaged spectrum of each sample was plotted in Fig. 5. The solid line is the averaged Raman spectra for cells at each GSK233470 concentration and the shade area is the ±1 standard deviation acquired for the test.

The Raman spectra of MCF7 cells showed intensity increase and decrease for certain bands when the GSK233470 concentration rose from 0 μM to 20 μM. Specifically, with GSK233470 concentration increasing, the Raman intensity decreased at 781 cm^{-1}, 1084 cm^{-1}, 1263 cm^{-1}, 1304 cm^{-1} and 1655 cm^{-1}, whilst increased at 1128 cm^{-1}. In addition, with the GSK233470 concentration increasing, the bands at 1579 cm^{-1} and 1616 cm^{-1} merged into 1601 cm^{-1}. The single-cell Raman spectrum intensity changed as a whole and demonstrated the changes of intracellular components.

Then we conducted the analysis on the Raman bands by R-studio software (Fig. 6). Compared with negative control (no D_2O, no GSK233470), the Raman band at 781 cm^{-1}, 1084 cm^{-1}, 1128 cm^{-1}, 1263 cm^{-1} and 1304 cm^{-1} bands were changed (increased or decreased) evidently with the increase of GSK233470 concentration (p<0.001). Apart from 1128cm^{-1}, significant intensity change was observed when the GSK233470 concentration rose up to 10 μM, which is consistent with the cytotoxicity value of the GSK233470 evaluated by the CCK8 assay and the Raman DIP method. The results revealed that drug-treated cells showed varying Raman bands intensity with the increasing of drug concentration. Such intensity change could possibly relate to the amount or density differences of the cellular components, such as the nucleic acid, protein, and lipid, which varied during the drug-induced cell death process. This spectral presentation was consistent with the fact that the chromatin of the nucleolus and other cellular components is highly fragmented in the oncosis process as the drug concentration increases.

Discussion

Here, we have used Raman-DIP method to study the response of the human breast adenocarcinoma cells (the MCF7 cells) to the targeted anticancer drug GSK233470 *in vitro*.

The PDK1 inhibitor cytotoxicity evaluated by Raman DIP method was highly consistent with that evaluated by the widely used CCK8 assay. The inhibition rates from Raman DIP method showed a correlation coefficient value (R^2= 0.88) with the CCK8 assay, indicating that the Raman DIP method could be as accurate as the CCK8 assay. Furthermore, the fingerprint range of the Raman spectra revealed the amount/density changes on cellular components in the MCF7 cell after the GSK233470 treatment, for the cellular component abundance varies during the drug-induced cell death. Compared with the negative control, the intensity of certain Raman bands in the fingerprint range showed significant changes when the GSK233470 concentration rose to 10 μM, which is consistent with the cytotoxicity value of the GSK233470 evaluated by the CCK8 assay and the Raman DIP method.

The fingerprint range Raman spectra between 400-1800cm^{-1} contains rich information about the intracellular proteins, nucleic acids, lipids, and other biological macromolecules, which reflects the physiological and biochemical status of the bio-sample. Previous study has reported Raman band intensity from cancerous nasopharyngeal tissues increased at 1268 cm^{-1}, 1579 cm^{-1} and 1660 cm^{-1} when compared to normal tissues, referring to higher quantity of proteins, lipids, and nucleic acids in the cancerous cells [16]. Our results showed that with the GSK233470 concentration increasing, the Raman intensity decreased at 781 cm^{-1} (cytosine/uracil ring breathing mode of nucleotide) [17], 1084 cm^{-1} (phosphodiester groups of nucleic acids) [18], 1263 cm^{-1}(thymine, adenine; =C-H bending mode of phospholipids) [16, 19, 20], 1304 cm^{-1} (CH_2 twisting mode of lipids, adenine, cytosine) [21] and 1655 cm^{-1} (C=O stretching mode of amide I proteins, α-helix conformation, C=C stretching mode of lipids) [21, 22]. Referring to the previous study, where the increased Raman band intensity related to higher quantity of intracellular components, our result showed intensity decreases from these relative Raman bands, indicating that the GSK233470 at certain concentrations can interrupt the synthesis of nucleic acid and protein in the MCF7 cells and reduce their intracellular

quantity. Meanwhile, the band intensity increased at 1128 cm^{-1} (C-N stretching mode of proteins, C-O stretching mode of carbohydrates, resonance Raman from cytochrome C) [22, 23], indicating the upregulation of the Cytochrome C. The Bcl2 family protein was known to affect cell apoptosis by activation or inactivation of mitochondrial outer membrane permeabilization pore, which is involved in regulation of Cytochrome C release into cytosol [24, 25]. Wang et at. found DT-13 treatment for prostate cancer can increase the expression level of Cytochrome C level in cytoplasm and trigger cell apoptosis [15]. The increased Raman band at 1128 cm^{-1} (Cytochrome C) could indicate that higher concentration of GSK233470 attributed to the blockade of Bcl-2/Akt pathway and the upregulation of the Cytochrome C, resulting in the cell apoptosis [15]. In addition, with the GSK233470 concentration increasing , the bands at 1579 cm^{-1} (C=C bending mode of phenylalanine) [16] and 1616 cm^{-1} (C=C stretching mode of tyrosine and tryptophan) [17] were combined to form a higher 1601 cm^{-1} band (C=C bending mode of phenylalanine and tyrosine) [22, 26]. Tryptophan is an α-amino acid that is used in the biosynthesis of proteins [27]. It can improve potassium or carbohydrate supplements [28] and increase co-enzymes such as pyridoxine or ascorbic acid synthesis [29]. Decreasing of tryptophan can leading to stunted growth. The Raman spectra showed a decrease on tryptophan and an increase on tyrosine, which possibly revealed that the growth of the MCF7 cells became stunted with the increase of the GSK233470 concentration.

Multiple in vitro tests are widely applied to assess the anticancer activity of new compounds. The MTT has been widely used and is considered as a gold standard for measuring cell viability and drug cytotoxicity. The assay is based on the assumption that MTT tetrazolium salt reduction to formazan occurs in the mitochondria of living cells due to the activity of mitochondrial dehydrogenases. However, use of MTT has proven inconsistent and nonspecific in many experimental circumstances. The MTT assay is significantly influenced

by compounds that modify cell metabolism by increasing the NADPH level or the activity of LDH. Furthermore, MTT tetrazolium salt may be reduced not only in the mitochondria but also within the cytoplasm, on the surface of cell, endosome or lysosome membranes, or even in the extracellular environment. Factors influencing the reduction process include the current phase of growth, the cell cycle phase, and reaction conditions such as pH and D-glucose concentration. A series of improved methods based on the principle of MTT method can overcome the shortcomings of MTT method, such as XTT, MTS, CCK8 and WST method. However, the four still have their own characteristics. XTT is not stable and is relatively difficult to dissolve. It needs to be heated when it is used to prepare liquid, so it is rarely used. MTS, CCK8 and WST are commercial kits, although the cost is relatively high, the experimental results are more objective and reliable. Whatever, all of these methods cannot avoid the edge effect that effect the accuracy and precision during cell culture. Raman spectroscopy, however, is a label-free and non-destructive approach. It provides intrinsic molecular profile of a single cell, and requires no complicated sample preparation nor expensive chemistry. With the D_2O stable isotope probing, Raman spectra can reveal the metabolism rate of the cancer cells and the inhibiting effects of the targeted drugs on the cancer cells at a single-cell le CCK8vel. Thus, Raman spectra can be a new method for evaluating targeted drugs on cancer cells.

Conclusion

Many studies have been performed using cultured cells with drug exposure to evaluate the chemotherapy effect through CCK8 assay in vitro. This study compared the cytotoxicity level of the GSK233470 to the MCF7 cell evaluated by Raman DIP method and CCK8 assay. The results from the two methods are highly matched, as both suggested that 10 μM was the cytotoxic concentration for cell regeneration inhibition. Thus, Raman DIP method can be

used to monitor the inhibition of the GSK233470 to the MCF7 cell proliferation with high accuracy as CCK8 assay. Furthermore, Raman signal can provide additional information on the bio-macromolecules of the cells from the fingerprint range. This study also showed that the variation of the bio-macromolecules reflected by Raman signal in the fingerprint range could also be used to evaluate the GSK233470 cytotoxicity.

This study shows that Raman DIP method can be an accurate method to evaluate the cytotoxicity of the GSK233470 to the MCF7 cells. The combination of Raman DIP results and fingerprint range information could effectively improve the evaluation, which can help to obtain a better result.

Acknowledgements

This work was supported by grant from the Chinese Academy of Sciences under Award Number ZDKYYQ20200004. Also acknowledges financial support from Jiangsu Shuangchuang Dorctor Award.

Author contributions

Huang Xia: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Project administration, Funding acquisition; **Li Chengjian:** Formal analysis, Investigation, Resources; **Jin Guilan:** Investigation, Resources; **Zhang Lihao:** Writing - Review & Editing; **Yi Xiaofei:** Investigation, Resources; **Huang Wei:** Conceptualization, Project administration, Supervision; **Zhao Liang:** Conceptualization, Methodology, Formal analysis, Visualization, Project administration, Supervision. All author contribute to draft edit and result discussion.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2019, 69 (2019) 7-34.

[2] R.M. Feng, Y.N. Zong, S.M. Cao, R.H. Xu, Current cancer situation in China: good or bad news from the 2018 Global Cancer Statistics?, Cancer communications (London, England), 39 (2019) 22.

[3] Z. Farhane, H. Nawaz, F. Bonnier, H.J. Byrne, In vitro label-free screening of chemotherapeutic drugs using Raman microspectroscopy: Towards a new paradigm of spectralomics, 11 (2018) e201700258.

[4] Y. Li, S. Su, Y. Zhang, S. Liu, H. Jin, Q. Zeng, L. Cheng, Accuracy of Raman spectroscopy in discrimination of nasopharyngeal carcinoma from normal samples: a systematic review and meta-analysis, Journal of Cancer Research and Clinical Oncology, 145 (2019) 1811-1821.

[5] A. Taketani, B.B. Andriana, H. Matsuyoshi, H. Sato, Raman endoscopy for monitoring the anticancer drug treatment of colorectal tumors in live mice, Analyst, 142 (2017) 3680-3688.

[6] Y. Wang, W.E. Huang, L. Cui, M. Wagner, Single cell stable isotope probing in microbiology using Raman microspectroscopy, Current Opinion in Biotechnology, 41 (2016) 34-42.

[7] Y. Tao, Y. Wang, S. Huang, P. Zhu, W. Huang, J.-Q. Ling, J. Xu, Metabolic-Activity-Based Assessment of Antimicrobial Effects by D2O-Labeled Single-Cell Raman Microspectroscopy, Analytical Chemistry, 89 (2017).

[8] O.O. Olaniyi, K. Yang, Y.-G. Zhu, L. Cui, Heavy water-labeled Raman spectroscopy reveals carboxymethylcellulose-degrading bacteria and degradation activity at the single-cell level, Applied Microbiology and Biotechnology, 103 (2019) 1455-1464.

[9] Y. Song, L. Cui, J. López, J. Xu, I. Thompson, W. Huang, Raman-Deuterium Isotope Probing for in-situ identification of antimicrobial resistant bacteria in Thames River, Scientific Reports, 7 (2017) 16648.

[10] M. Diem, A. Mazur, K. Lenau, J. Schubert, B. Bird, M. Miljković, C. Krafft, J. Popp, Molecular pathology via IR and Raman spectral imaging, J Biophotonics, 6 (2013) 855-886.

[11] B. Vanhaesebroeck, D. Alessi, The PI3K-PDK1 connection: more than just a road to PKB, The Biochemical journal, 346 Pt 3 (2000) 561-576.

[12] V. Kirkin, S. Joos, M. Zörnig, The role of Bcl-2 family members in tumorigenesis, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1644 (2004) 229-249.

[13] R. Arsenic, Immunohistochemical analysis of PDK1 expression in breast cancer, Diagnostic pathology, 9 (2014) 82.

[14] A. Najafov, E.M. Sommer, J.M. Axten, M.P. Deyoung, D.R. Alessi, Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1, Biochem J, 433 (2011) 357-369.

[15] Z. Wang, Y. Wang, S. Zhu, Y. Liu, X. Peng, S. Zhang, Z. Zhang, Y. Qiu, M. Jin, R. Wang, Y. Zhong, D. Kong, DT-13 Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells Through Blocking PI3K/Akt Pathway, Frontiers in pharmacology, 9 (2018) 1450.

[16] Y. Li, J. Pan, G. Chen, C. Li, S. Lin, Y. Shao, S. Feng, Z. Huang, S. Xie, H. Zeng, R. Chen, Micro-Raman spectroscopy study of cancerous and normal nasopharyngeal tissues, Journal of biomedical optics, 18 (2013) 27003.

[17] N. Stone, C. Kendall, N. Shepherd, P. Crow, H. Barr, Near-infrared Raman spectroscopy for the classification of epithelial pre-cancers and cancers, 33 (2002) 564-573.

[18] R.K. Dukor, Vibrational Spectroscopy in the Detection of Cancer, Handbook of Vibrational Spectroscopy2001.

[19] S. Qiu, Q. Huang, L. Huang, J. Lin, J. Lu, D. Lin, G. Cao, C. Chen, J. Pan, R. Chen, Label-free discrimination of different stage nasopharyngeal carcinoma tissue based on Raman spectroscopy, Oncol Lett, 11 (2016) 2590-2594.

[20] J.W. Chan, D.S. Taylor, T. Zwerdling, S.M. Lane, K. Ihara, T. Huser, Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells, Biophysical journal, 90 (2006) 648-656.

[21] N. Stone, C. Kendall, J. Smith, P. Crow, H. Barr, Raman spectroscopy for identification of epithelial cancers, Faraday Discussions, 126 (2004) 141-157.

[22] S.-i. Morita, S. Takanezawa, M. Hiroshima, T. Mitsui, Y. Ozaki, Y. Sako, Raman and Autofluorescence Spectrum Dynamics along the HRG-Induced Differentiation Pathway of MCF-7 Cells, Biophysical Journal, 107 (2014) 2221-2229.

[23] R. Pätzold, M. Keuntje, K. Theophile, J. Müller, E. Mielcarek, A. Ngezahayo, A. Anders-von Ahlften, In situ mapping of nitrifiers and anammox bacteria in microbial aggregates by means of confocal resonance Raman microscopy, Journal of Microbiological Methods, 72 (2008) 241-248.

[24] N.N. Danial, BCL-2 family proteins: critical checkpoints of apoptotic cell death, Clinical cancer research : an official journal of the American Association for Cancer Research, 13 (2007) 7254-7263.

[25] E.A. Slee, M.T. Harte, R.M. Kluck, B.B. Wolf, C.A. Casiano, D.D. Newmeyer, H.G. Wang, J.C. Reed, D.W. Nicholson, E.S. Alnemri, D.R. Green, S.J. Martin, Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner, The Journal of cell biology, 144 (1999) 281-292.

[26] N. Stone, P. Stavroulaki, C. Kendall, M. Birchall, H. Barr, Raman Spectroscopy for Early Detection of Laryngeal Malignancy: Preliminary Results, 110 (2000) 1756-1763.

[27] Nomenclature and Symbolism for Amino Acids and Peptides, 138 (1984) 9-37.

[28] A. Coppen, D.M. Shaw, B. Herzberg, R. Maggs, Tryptophan in the treatment of depression, Lancet (London, England), 2 (1967) 1178-1180.

[29] R.N. Herrington, A. Bruce, E.C. Johnstone, M.H. Lader, COMPARATIVE TRIAL OF L-TRYPTOPHAN AND E.C.T. IN SEVERE DEPRESSIVE ILLNESS, The Lancet, 304 (1974) 731-734.

Fig. 1. MCF7 cells visibilities at different GSK233470 concentrations.

Fig.2. White light images of MCF7 cells after GSK233470 treatment

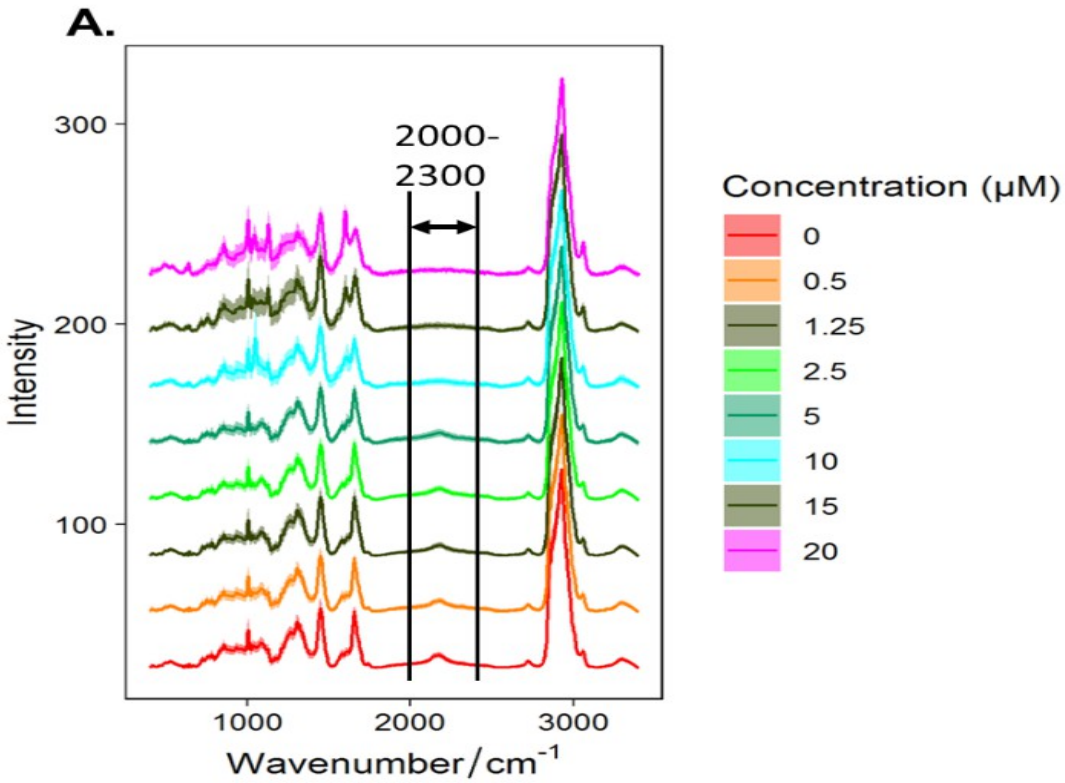


Fig. 3. Raman result of the MCF7 cells after GSK233470 treatment and 48-hour D_2O incubation. (A) Raman spectra of the MCF7 cells. (B) The C-D/(C-H+C-D) ratio of each Raman spectra.

Fig.4.Compare inhibition rates from Raman DIP results and CCK8 assay results

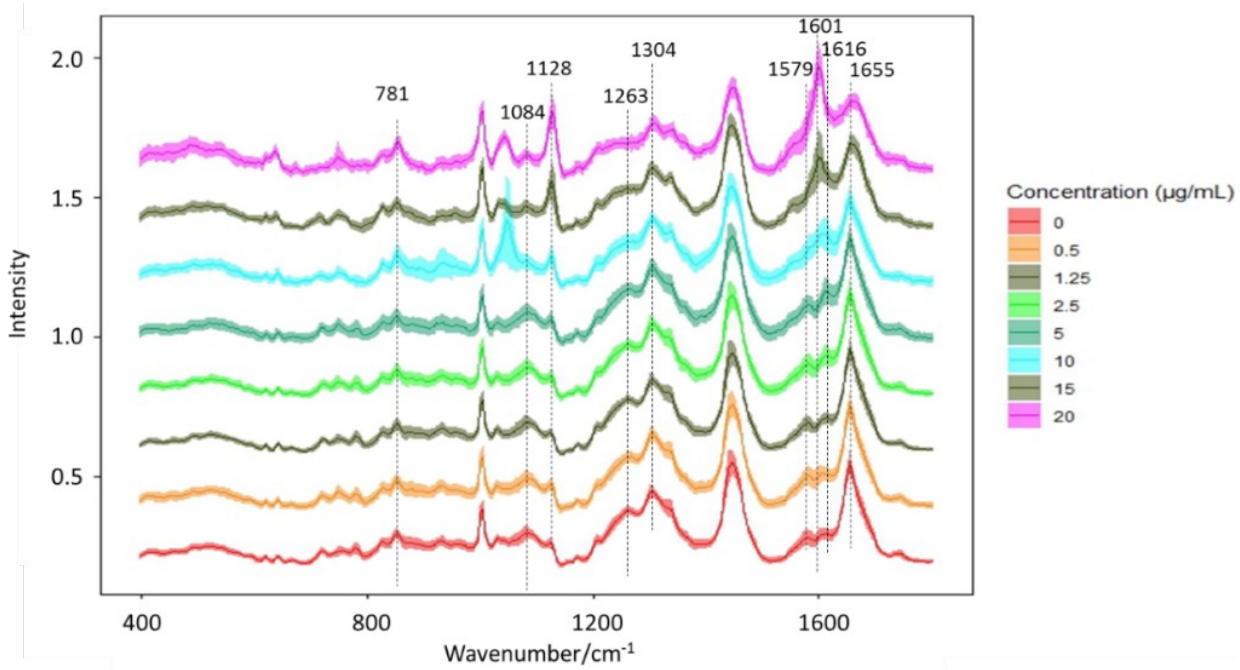
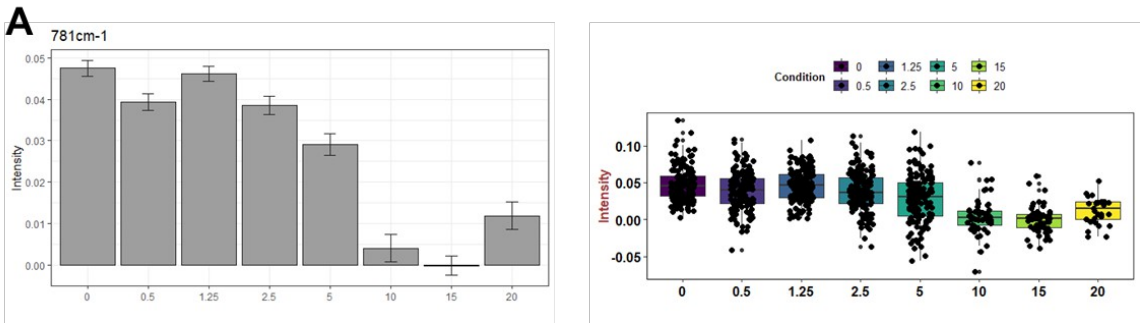
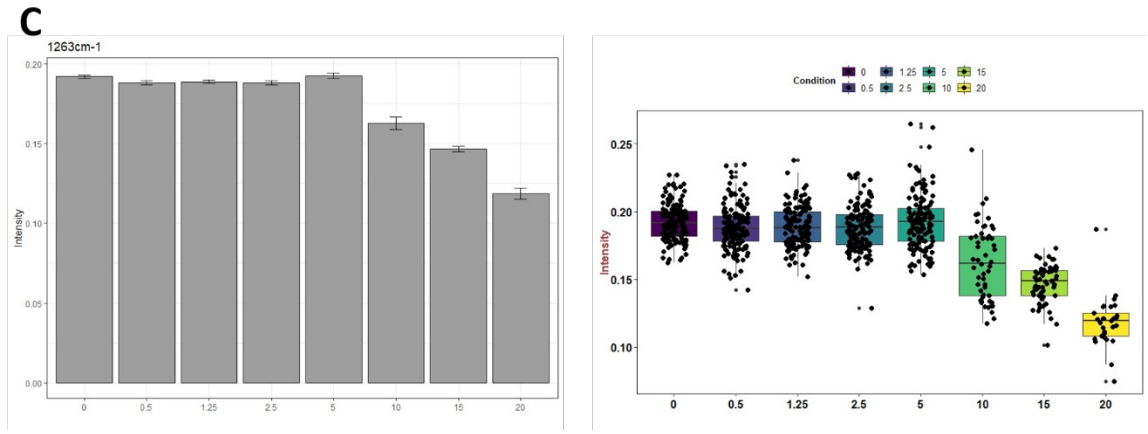
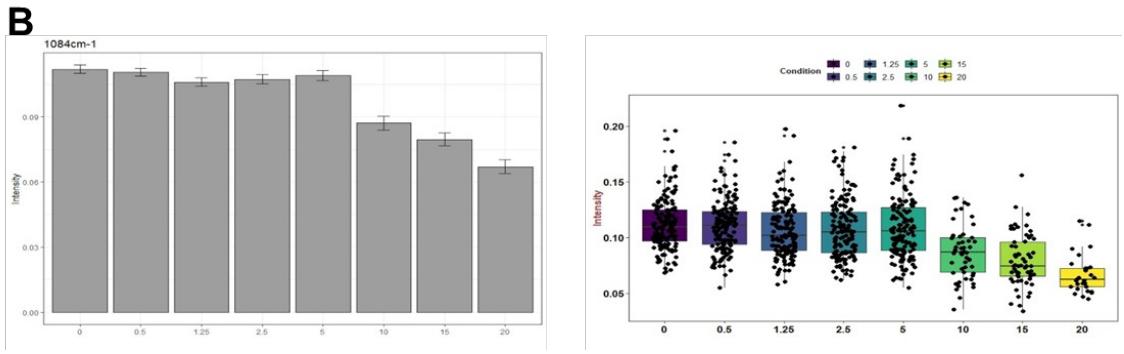


Fig. 5 The fingerprint range Raman spectra of MCF7 cells at different GSK233470 concentrations.


A
781cm-1
Intensity
0.05
0.04
0.03
0.02
0.01
0.00
0
0.5
1.25
2.5
5
10
15
20
Condition
0
0.5
1.25
2.5
5
10
15
20
Intensity
0.10
0.05
0.00
-0.05


C
1263cm-1
Intensity
0.20
0.15
0.10
0.05
0.00
0
0.5
1.25
2.5
5
10
15
20
Condition
0
0.5
1.25
2.5
5
10
15
20
Intensity
0.25
0.20
0.15
0.10


B
1084cm-1
Intensity
0.09
0.06
0.03
0.00
0
0.5
1.25
2.5
5
10
15
20
Condition
0
0.5
1.25
2.5
5
10
15
20
Intensity
0.20
0.15
0.10
0.05

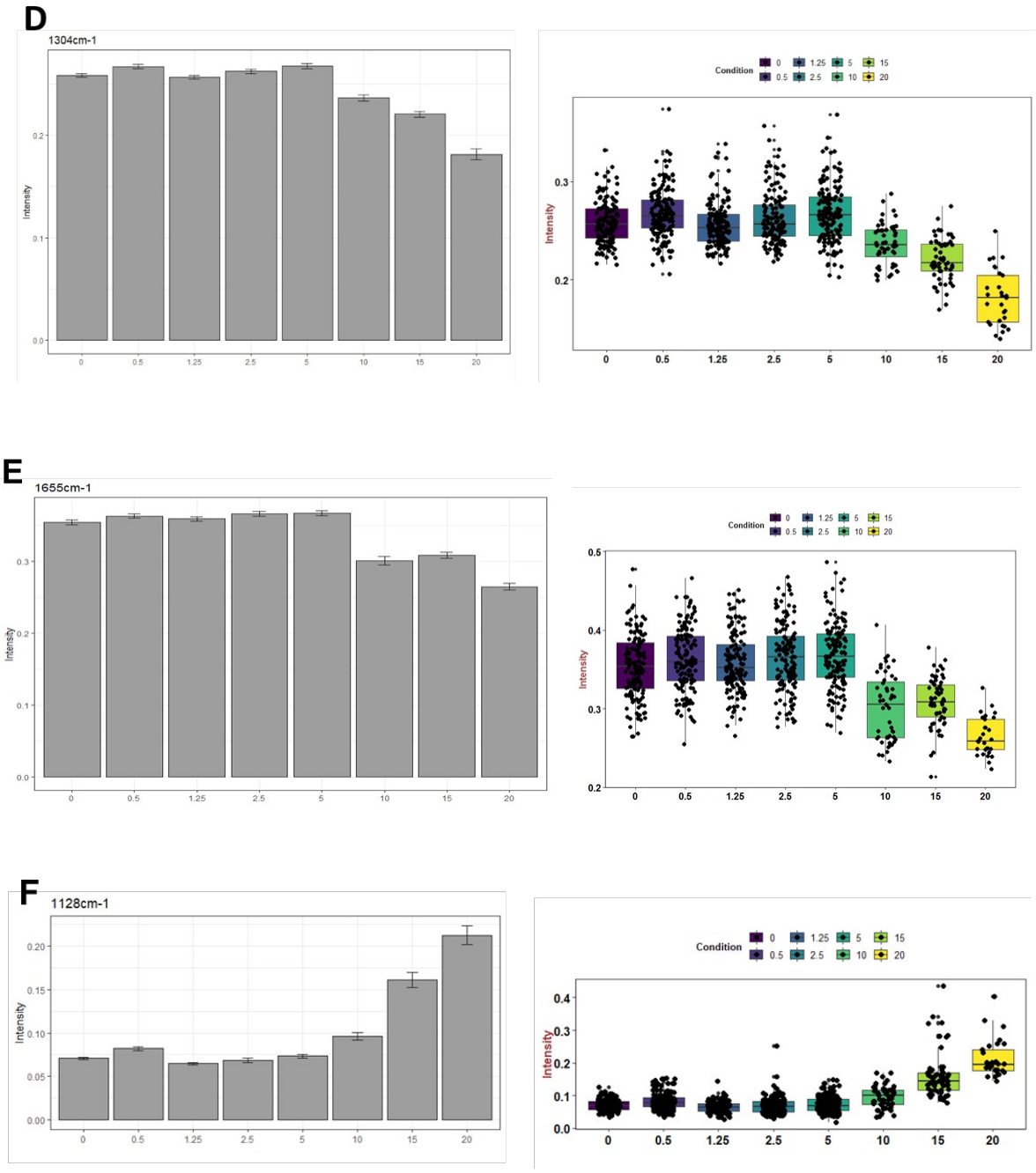


Fig.6. Raman band intensity at different GSK233470 concentration comparing with negative control (no D_2O, no GSK233470).