Journal of Biomedical Optics

SPIEDigitalLibrary.org/jbo

Optical molecular imaging approach for rapid assessment of response of individual cancer cells to chemotherapy

Zhen Luo
Rohan Vijay Tikekar
Kiana Michelle Samadzadeh
Nitin Nitin

SPIE

Optical molecular imaging approach for rapid assessment of response of individual cancer cells to chemotherapy

Zhen Luo,[a] Rohan Vijay Tikekar,[b,c] Kiana Michelle Samadzadeh,[a] and Nitin Nitin[a,b]
[a]University of California-Davis, Department of Biological and Agricultural Engineering, Old Davis Road, Davis, California 95616
[b]University of California-Davis, Food Science and Technology Department, One Shields Avenue, Davis, California 95616
[c]Drexel University, Program in Food Science, 101 North 33rd Street, Philadelphia, Pennsylvania 19104

Abstract. Predicting the response of individual patients to cytotoxic chemotherapy drugs is critical for developing individualized therapies. With this motivation, an optical molecular imaging approach was developed to detect cisplatin induced changes in the uptake and intracellular retention of choline. Intracellular uptake of choline was characterized using a click chemistry reaction between propargyl choline and Alexa-488 azide. Cisplatin induced changes in the uptake of propargyl choline in cells and tumor spheroids were compared with similar measurements using a fluorescent analogue of deoxyglucose and conventional cell viability assays. Uptake and intracellular retention of propargyl choline decreased with an increase in concentration of cisplatin. Intracellular uptake of propargyl choline was significantly reduced within 3 h of incubation with a sub-lethal dose of cisplatin. Results demonstrate that the imaging approach based on propargyl choline was more sensitive in detecting the early response of cancer cells to cisplatin as compared to the imaging based on fluorescent analogue of deoxyglucose and cell viability assays. Imaging measurements in tumor spheroids show a significant decrease in the uptake of propargyl choline following treatment with cisplatin. Overall, the results demonstrate a novel optical molecular imaging approach for rapid measurement of the response of individual cancer cells to cisplatin treatment. [DOI: 10.1117/1.JBO.17.10.106006]

Keywords: fluorescence; imaging; materials; medical imaging.

Paper 12283 received May 9, 2012; revised manuscript received Aug. 30, 2012; accepted for publication Aug. 31, 2012; published online Oct. 1, 2012.

1 Introduction

Personalized therapy can significantly improve the therapeutic outcome for cancer patients.[1] Molecular imaging technologies can enable personalized selection and evaluation of therapies.[2–4] Based on this personalized approach, clinicians can select optimal therapies and adjust the dosage and frequency of the selected therapies for individual patients. With this motivation, the focus of this study was to develop and validate a novel optical molecular imaging approach for evaluating the response of cancer cells to cisplatin treatment. In this study, cisplatin was selected as a model platinum-based chemotherapy drug as it is widely used in clinical practice for treating diverse cancers including ovarian, head and neck, lung, and breast cancer.[5–7] The optical molecular imaging approach was based on measuring cisplatin induced changes in the uptake and intracellular retention of choline. Intracellular retained propargyl choline was fluorescently labeled using a click chemistry reaction between propargyl choline and Alexa-488 azide.

The optical molecular imaging approach to measure choline uptake in cells was based on the understanding that the increased rate of membrane synthesis in rapidly dividing cancer cells results in upregulation of choline transporters and choline kinase enzymes.[8–10] These biochemical changes increase the rate of choline uptake (an essential component of cell membrane) in cancer cells.[11] Studies have also shown that successful cancer therapies decrease the rate of uptake and intracellular retention of choline.[12] This decrease in the rate of uptake of choline by cancer cells has formed the basis for ^{11}C-choline positron emission tomography (PET) imaging to assess the response of tumors to cancer therapies.[12,13] Similarly, therapy induced changes in choline metabolism of cancer tissues has formed the basis for magnetic resonance (MR) spectroscopy approaches to evaluate the response of tumors to cancer therapies.[14,15] Although both PET and MR imaging methods have significant clinical advantages for deep tissue imaging, both methods have relatively low spatial resolution. The low spatial resolution of PET and MR imaging technologies[16,17] limits detection of heterogeneity within tumors[18] and reduces sensitivity for measuring the early response of cancer cells to therapies.[19,20] Optical molecular imaging is a complementary imaging technology that has the potential to address some of the limitations of PET and MR imaging technologies.

This study was focused on developing a novel optical molecular imaging approach based on measuring the changes in metabolic activity of individual cells induced by cisplatin. In this study, analogues of two distinct metabolites (glucose and choline) were compared to evaluate the response of cancer cells and 3-d spheroids to cisplatin treatment. Decrease in the uptake of fluorescently labeled deoxyglucose analogue with drug treatment was recently demonstrated in ovarian[21] and breast cancer cells.[22] The fluorescent analogue of deoxyglucose molecule (2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose) is an optical analogue of radio labeled FDG molecule that is routinely used in clinical practice for both cancer detection and evaluation of therapy response.

Address all correspondence to: Nitin Nitin, 2214 Robert Mondavi Institute South, Old Davis Road, Davis, California 95616; Tel: 530-752-6208; Fax: 530-752-2640; E-mail: nnitin@ucdavis.edu.

0091-3286/2012/$25.00 © 2012 SPIE

The results of the optical molecular imaging measurements were compared with the conventional cell viability [MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole)] and apoptosis (PI/Annexin V staining) assays to evaluate sensitivity of the optical imaging approaches for measuring the response of cancer cells to cisplatin treatment.

2 Methods and Materials

2.1 *Chemicals*

A 10 mM stock solution of cisplatin (Sigma-Aldrich, St. Louis, MO) was prepared in distilled water. 2-NBDG was purchased from Invitrogen (Life Technologies, Grand Island, NY). A 5 mg/mL stock solution of 2-NBDG was prepared in distilled water. MTT cell proliferation assay kit was purchased from ATCC (Manassas, VA). Propargyl choline was synthesized using the method described by Jao and others.[23] 4 g propargyl bromide (80% solution in toluene) was added to 3 g of dimethylethanolamine in 10 mL tetrahydrofuran. The mixture was stirred over ice for approximately 30 min. The reaction mixture was then stirred for 24 h under nitrogen. Propargyl choline (white solid) was separated from the solvent by filtration (Millipore filter) followed by repeated washing with tetrahydrofuran. The dried product was stored at −20°C.

2.2 *Cell Culture*

Human cervical carcinoma cell line, HeLa, was maintained in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific, Pittsburgh, PA) supplemented with 10% FBS (Fisher Scientific, Pittsburgh, PA) and 100 mg/L penicillin (Sigma, St Luois, MO). HeLa cells (4×10^4 cells/mL) were seeded into culture flasks, grown in a humidified atmosphere of 5% CO_2–95% air at 37°C, and subcultured with 0.05% trypsin (Invitrogen). For fluorescence imaging, cells were cultured on 8 well cover slip bottom culture chambers (Fisher).

2.3 *Multicellular Spheroid Formation*

The liquid-overlay technique (hanging drop method) was used to form multicellular spheroids.[24] Cells from a confluent T-25 flask were detached using trypsin-EDTA and resuspended in a culture medium to a final concentration of 10^7 cells/mL. To form individual droplets, 20 μL of the concentrated cell suspension was deposited on the lid of a Petri dish. The bottom chamber of a Petri dish was filled with 2 mL of cell culture medium to limit evaporation of moisture from individual droplets. The lid with individual droplets was inverted over a Petri dish to form hanging drops. The Petri dish was placed in an incubator for a period of 5 to 7 days, after which visible cell spheroids were formed in individual hanging drops. To treat individual droplets with cisplatin, an approximately 2 μL of cisplatin stock solution (1 mM) was injected using a micropipette into an individual hanging droplet. Cisplatin concentration in individual droplets was approximately 100 μM. Individual hanging drops were treated with cisplatin for 48 h prior to incubation with propargyl choline or 2-NBDG.

2.4 *Choline Uptake Assay*

HeLa cells were treated with cisplatin at selected concentration levels (0 to 100 μM) for a specified incubation time (ranging from 0 to 96 h). After the drug treatment, cells were incubated with propargyl choline (2 mM in regular cell culture medium) for 1 h at 37°C. Propargyl choline labeled cells were rinsed with PBS and fixed using formaldehyde (3.7% in PBS) at 4°C. After 30 min of fixation, cells were rinsed with PBS and incubated with the click chemistry reaction buffer for 30 min in the dark at room temperature. This reaction buffer contains 0.1 M Tris-Buffer (pH = 8.5), 0.05% Triton, 10 μM Alexa-488 azide, 1 mM $CuSO_4$, and 50 mM Ascorbic Acid. After the click chemistry reaction, cells were rinsed and imaged using an inverted fluorescence microscope (IX71, Olympus Inc., Center Valley, PA). Excitation and emission filters for fluorescence microscopy were 470/15 nm and 520 to 550 nm, respectively. Exposure time of the CCD camera (charge-coupled device, Hamamatsu Photonics, Bridgewater, NJ) was 500 ms for fluorescence imaging of cells. Multicellular spheroids were treated with a fixed concentration of cisplatin for 48 h prior to incubation with propargyl choline using the same procedure as described above for the 2-d cell culture models. Multicellular spheroids were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with 488 nm laser excitation and a band pass emission filter (520 to 550 nm).

2.5 *Glucose Uptake Assay*

Changes in intracellular uptake of 2-NBDG following the incubation of cells with cisplatin were measured using similar cell culture and incubation procedures as described in the choline uptake assay. The key differences in the glucose uptake assay as compared to the choline uptake assay were the incubation time (30 min of incubation time for 2-NBDG), use of glucose free DMEM during incubation of cells with 2-NBDG and imaging of cells and spheroids labeled with 2-NBDG without chemical fixation. The incubation time was selected based on the results of our previous study.[21]

2.6 *Quantification of Imaging Data*

To quantify imaging data, the mean fluorescence intensity (MFI) of individual cells within the field of view (FOV) was calculated using ImageJ (Public domain, NIH). The MFI of individual cells was corrected by subtracting the background fluorescence signal from a region on a chamber cover slip without any cells. It is important to note that the fluorescence intensity values corresponding to both background fluorescence and cellular autofluorescence were significantly small as compared to the fluorescence signal intensity from propargyl choline or 2-NBDG stained cells. Multiple FOVs (typically 9 to 10 FOVs with approximately 90 to 120 cells in total) were analyzed to calculate the MFI from three independent experiments for each of the experimental conditions. Average MFI and standard deviation were calculated for each experimental condition.

2.7 *Statistical Analysis*

Statistical analysis was carried out using Microsoft® Excel 2007 (Microsoft Inc., Bellevue, WA) and SAS® (version 9.1 SAS Inc., Cary NC). Student's *t*-test or ANOVA was used for evaluating statistical significance between the treatments with a 95% confidence level. Differences between the means were evaluated with Duncan's *t*-test and differences were considered to be significant if the p values were less than 0.05.

2.8 *MTT Assay*

For the MTT cell proliferation, cells cultured in 24-well plates were treated with cisplatin at selected cisplatin concentrations and for specified time (24 to 96 h). After treatment with cisplatin, the MTT assay was conducted according to the instructions of the MTT Cell Proliferation Assay (ATCC, Manassas, VA). Briefly, 10 μL of the MTT reagent was added to individual wells and the plates were incubated for 3 h at 37°C. After 3 h, 100 μL of the detergent reagent (supplied with the kit) was added to individual wells of the plate. The plate was then incubated overnight in the dark at room temperature. Absorbance values at 570 nm were measured from individual wells using a plate reader (Synergy2, Bio Tek Inc., Winooski, VT). The MTT measurements were conducted in triplicate and the experiment was repeated three times for statistical comparison of the data.

2.9 *Apoptosis Assay*

For imaging apoptosis, cells were cultured on chamber cover slips and treated with cisplatin (1 and 10 μM) for 24 h. Cisplatin treated cells were washed and incubated with 1 × assay buffer containing Annexin V fluorescein isothiocyanate and propidium iodide (PI) according to the instructions from the vendor [Annexin V apoptosis detection kit (sc-4252 AK), Santa Cruz Biotechnology, Santa Cruz, CA]. In this assay, the binding of fluorescently labeled Annexin V to the cell membrane indicates changes in membrane structure and is a marker for the early stages of apoptosis. Permeation of PI dye (DNA binding dye) across the cell membrane and staining of the nuclei indicate damage to cell membranes induced by cisplatin treatment. PI staining is a marker for the late stages of apoptosis.[25] Together, the combination of these agents provides a comprehensive and an established approach to measure apoptosis in cells induced by drug treatment.

3 Results

3.1 *Effect of Cisplatin Treatment on Propargyl Choline Uptake*

Cisplatin induced changes in intracellular uptake of choline were measured using fluorescence imaging. For fluorescence imaging, an analogue of choline (propargyl choline) with a monoalkyne group at the N-terminus of a choline molecule was synthesized as described in the materials and methods section. The click-chemistry reaction between Alexa-488 azide and monoalkyne modified choline (propargyl choline) molecules enabled *in-situ* detection of propargyl choline in cells.

Fluorescence imaging measurements in Fig. 1(a) show a decrease in the intracellular uptake of propargyl choline with an increase in concentration of cisplatin (0 to 100 μM). In this experiment, HeLa cells were treated with cisplatin (concentration range from 0 to 100 μM) for 24 h prior to incubation with propargyl choline (for 1 h). To quantify the imaging data, the MFI from individual cells was calculated and normalized with respect to the positive control (propargyl choline labeled cells without drug treatment). Figure 1(b) shows changes in the normalized fluorescence intensity as a function of cisplatin concentration (average of three independent measurements). The results show that intracellular uptake of propargyl choline decreased with an increase in concentration of cisplatin. Treatment of cells with 10 μM of cisplatin concentration resulted in approximately 60% decrease in the uptake of propargyl choline in cells after 24 h. At higher concentration levels (50 to 100 μM of cisplatin), the mean fluorescence signal intensity decreased by more than 90%.

The specificity of the click chemistry reaction was validated using a negative control [Fig. 1(c)]. In this control experiment, cells were labeled with Alexa 488-azide without prior incubation with propargyl choline. The results of the fluorescence imaging measurements show no significant fluorescence staining in the negative control cells [Fig. 1(c)] while significant

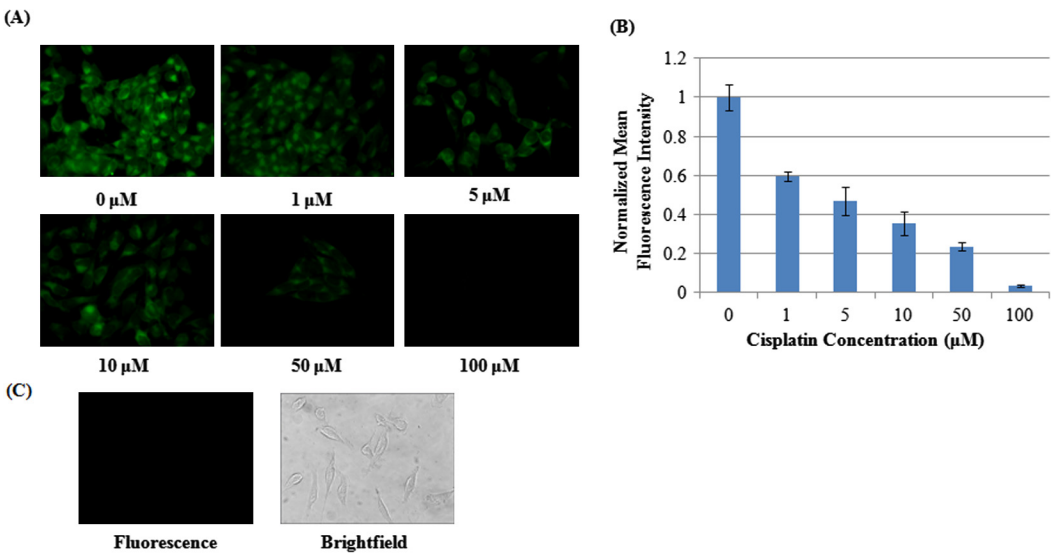

Fig. 1 Changes in uptake and intracellular retention of propargyl choline in cells as a function of cisplatin concentration. (a) Fluorescence images of HeLa cells treated with cisplatin at the indicated concentrations for 24 h. (b) Normalized fluorescence signal intensity of HeLa cells labeled with propargyl choline after 24 h of cisplatin treatment. (c) Fluorescence and corresponding brightfield images show high specificity of click chemistry labeling of HeLa cells labeled with Alexa-488 azide without incubation of cells with propargyl choline.

fluorescence staining was observed in the positive control cells [Fig. 1(a): cells without cisplatin treatment]. This result demonstrates specificity of the click chemistry reaction in cells.

The specificity of intracellular uptake of propargyl choline was also demonstrated using a competition assay (described in the supplementary information section). A fixed concentration of propargyl choline was coincubated with an increasing concentration of unmodified choline for 1 h in a cell culture model. The results of this competition assay show that the uptake of propargyl choline in cells decreased with an increase in concentration of unmodified choline, indicating that propargyl choline was specifically uptaken by cells through choline transporters.

3.2 *Effect of Cisplatin Treatment on 2-NBDG Uptake*

Figure 2(a) shows a decrease in the uptake of 2-NBDG with an increase in concentration of cisplatin. This trend is similar to the results in Fig. 1(a). Treatment of cells with 10 μM of cisplatin concentration resulted in approximately a 40% decrease in the uptake of 2-NBDG in cells after 24 h [Fig. 2(b)]. Comparison between the results in Figs. 2(b) and 1(b) demonstrates that the uptake of choline was reduced more than the uptake of 2-NBDG upon treatment with cisplatin.

3.3 *Effect of Cisplatin Treatment on Cell Viability*

Cell viability of HeLa cells treated with cisplatin for 24 and 96 h was measured using the MTT assay. The MTT assay measures the activity of mitochondrial reductase enzymes and is a well-established assay to measure cell viability. The results of the MTT assay are shown in Fig. 3(a). These results show that cell viability was not significantly reduced with exposure to low concentration levels of cisplatin (1 to 10 μM) for both 24 and 96 h incubation time. A significant decrease in cell viability was observed only at higher concentration levels of cisplatin (above 25 μM). These results are in agreement with previously published reports[21,26] and these results highlight that although cells were viable based on the MTT assay, significant changes in the uptake and intracellular retention of both propargyl choline and 2-NBDG were detected. This suggests that changes in the intracellular flux of metabolites are significantly influenced by cisplatin prior to induction of cell death (measured based on the MTT assay).

To detect apoptosis, HeLa cells were treated with 0, 1, and 10 μM of cisplatin for 24 h and stained using PI/Annexin V [Fig. 3(b)]. The results show no significant increase in Annexin V staining in cisplatin treated cells at 1 μM concentration as compared to the controls (cells not treated with cisplatin). It is also important to note that nonspecific binding of Annexin V to the cell membrane results in significant background fluorescence staining in the control cells. Similarly, no significant PI staining was detected in cells treated with 1 μM of cisplatin. In the case of HeLa cells treated with 10 μM of cisplatin, a significant increase in the number of cells stained with PI and Annexin V was observed as compared to the control cells. These results indicate that PI/Annexin V staining is more sensitive than the MTT assay in detecting the response of cells to cisplatin treatment, but it has

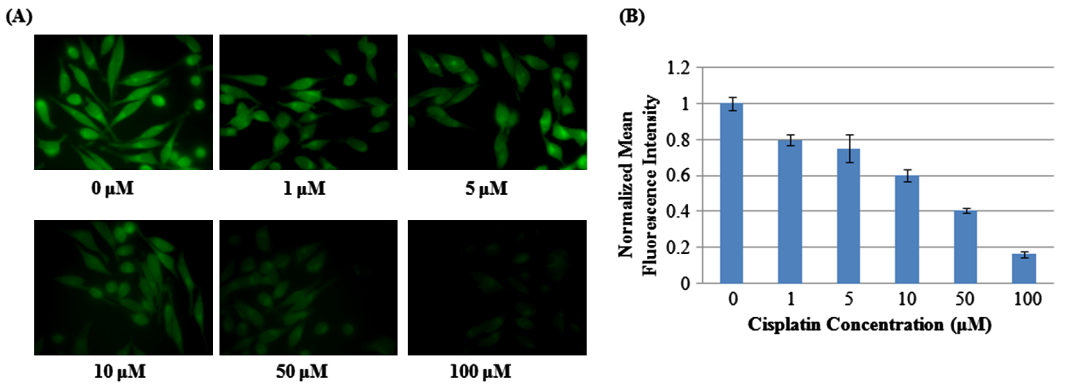

Fig. 2 Changes in uptake and intracellular retention of 2-NBDG in cells as a function of cisplatin concentration. HeLa cells were treated with cisplatin at the indicated concentrations for 24 h. (a) Fluorescence images show decrease in uptake of 2-NBDG with increasing concentration of cisplatin. (b) Normalized fluorescence signal intensity of HeLa cells labeled with 2-NBDG after 24 h of cisplatin treatment.

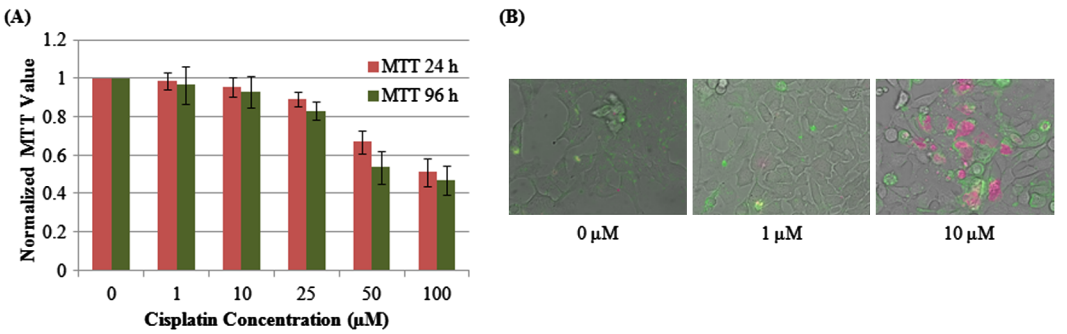

Fig. 3 Conventional cell viability assays to evaluate the response of HeLa cells to cisplatin treatment. (a) Cell viability assessed by MTT assay after 24 or 96 h of cisplatin treatment. (b) Brightfield/fluorescence overlay images of HeLa cells treated with cisplatin at indicated concentration for 24 h and stained with PI/Annexin V mixture. Annexin V positive cells (green) and PI staining cells (red) indicates the early and late stages of cell apoptosis induced by cisplatin.

limited sensitivity as compared to changes in the uptake and intracellular retention of propargyl choline and 2-NBDG.

3.4 *Early Decrease of Propargyl Choline and 2-NBDG Uptake after Cisplatin Treatment*

The next goal was to characterize cisplatin-induced changes in the uptake of propargyl choline and 2-NBDG in cells as a function of time. For these experiments, a sub-lethal dosage of cisplatin (1 μM) was selected to demonstrate sensitivity of the imaging approaches. This sub therapeutic concentration of cisplatin (1 μM) was at least 50 times lower than the IC-50 value determined using the MTT assay. Changes in the uptake of propargyl choline in HeLa cells were measured every 3 h. This time interval of 3 h was selected based on the results of a prior study.[21] The results of these measurements [Fig. 4(b)] show a significant decrease in the uptake of propargyl choline (approximately 55%, $p = 0.032$) during the first 3 h of incubation of cells with cisplatin at 1 μM. These changes in the uptake of propargyl choline precede detection of loss in cell viability by the MTT assay and the PI/Annexin V staining. It is expected that with an increase in concentration of cisplatin, the time required to detect changes in the uptake of propargyl choline can be further reduced.

Figure 5 characterizes the changes in the uptake of 2-NBDG in HeLa cells treated with 1 μM cisplatin as a function of time. Figure 5(b) shows the changes in the normalized MFI of 2-NBDG labeled cells as a function of incubation time with cisplatin. The results show that approximately 6 h of drug incubation time (with 1 μM of cisplatin) was required to achieve a significant decrease (approximately 36%, $p = 0.043$) in the intracellular uptake of 2-NBDG. These results in Figs. 4 and 5 demonstrate that cisplatin induced changes in the uptake of propargyl choline were rapid and more pronounced as compared to cisplatin induced changes in the uptake of 2-NBDG.

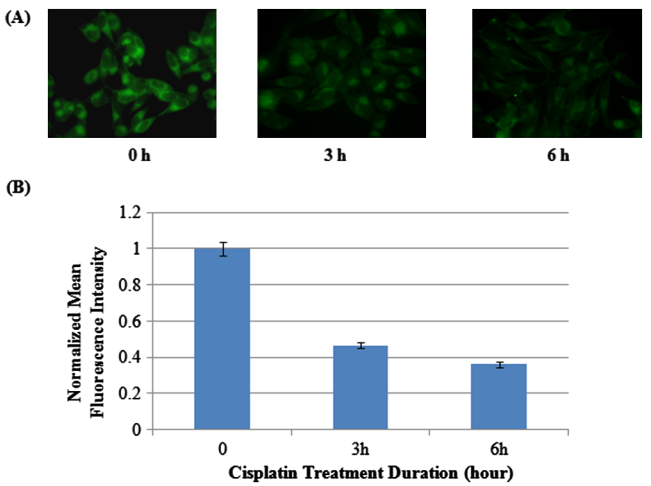

Fig. 4 Changes in uptake and intracellular retention of propargyl choline in cells as a function of cisplatin incubation time. HeLa cells were treated with cisplatin at the concentration of 1 μM. (a) Fluorescence images show changes in fluorescence intensity as a function of time (over 6 h) of cisplatin treatment. (b) Changes in normalized mean fluorescence signal intensity of HeLa cells treated with cisplatin as a function of time. Cells were labeled with propargyl choline after cisplatin treatment for a defined period of time.

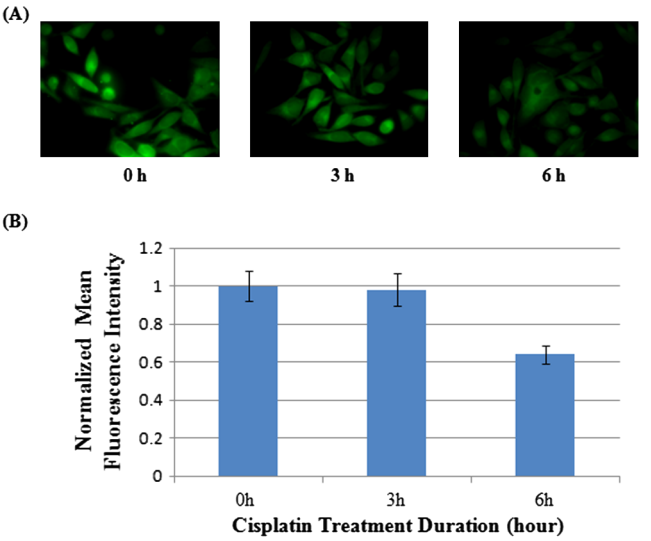

Fig. 5 Changes in uptake and intracellular retention of 2-NBDG in cells as a function of cisplatin incubation time. HeLa cells were treated with cisplatin at the concentration of 1 μM. (a) Fluorescence images show change in fluorescence intensity as a function of time (over 6 h) of cisplatin treatment. (b) Changes in normalized mean fluorescence signal intensity of HeLa cells treated with cisplatin as a function of time. Cells were labeled with 2-NBDG after cisplatin treatment for a defined period of time.

3.5 *Cisplatin Treatment in Multicellular Tumor Spheroid Model*

Multicellular tumor spheroids mimicking the structural features of a solid avascular tumor core were formed using the liquid-overlay method as described in the materials and methods section. The results in Fig. 6 characterize the uptake of propargyl choline and 2-NBDG in 3-d tumor spheroids treated with cisplatin (concentration of 100 μM for 48 h) as described in the materials and methods section. The results were compared with control spheroids without cisplatin treatment.

The optical imaging measurements [Fig. 6(a)] in control spheroids demonstrate uniform uptake of propargyl choline. After treatment with cisplatin, a significant decrease in intracellular uptake of propargyl choline was observed. The results in Fig. 6(b) characterize the uptake of 2-NBDG in 3-d tumor spheroids (control and drug treated samples). These results illustrate uniform delivery of 2-NBDG in control tumor spheroids and a significant decrease in uptake of 2-NBDG in tumor spheroids following treatment with cisplatin. These results demonstrate that the imaging approaches have a significant potential to detect the response of individual cells to cisplatin treatment in a 3-d tumor model.

4 Discussion

The goal of this study was to develop an optical molecular imaging approach for the rapid assessment of drug response in cancer cells and 3-d tumor spheroids. This imaging approach was based on measuring reduction in the uptake of choline in cancer cells upon treatment of cells with cisplatin. To image the uptake of choline in individual cells, a monoalkyne derivative of choline (propargyl choline) was synthesized. Intracellular uptake of this analogue was measured by in-situ fluorescent labeling of propargyl choline using a click chemistry reaction between Alexa-488 azide and propargyl choline. The results

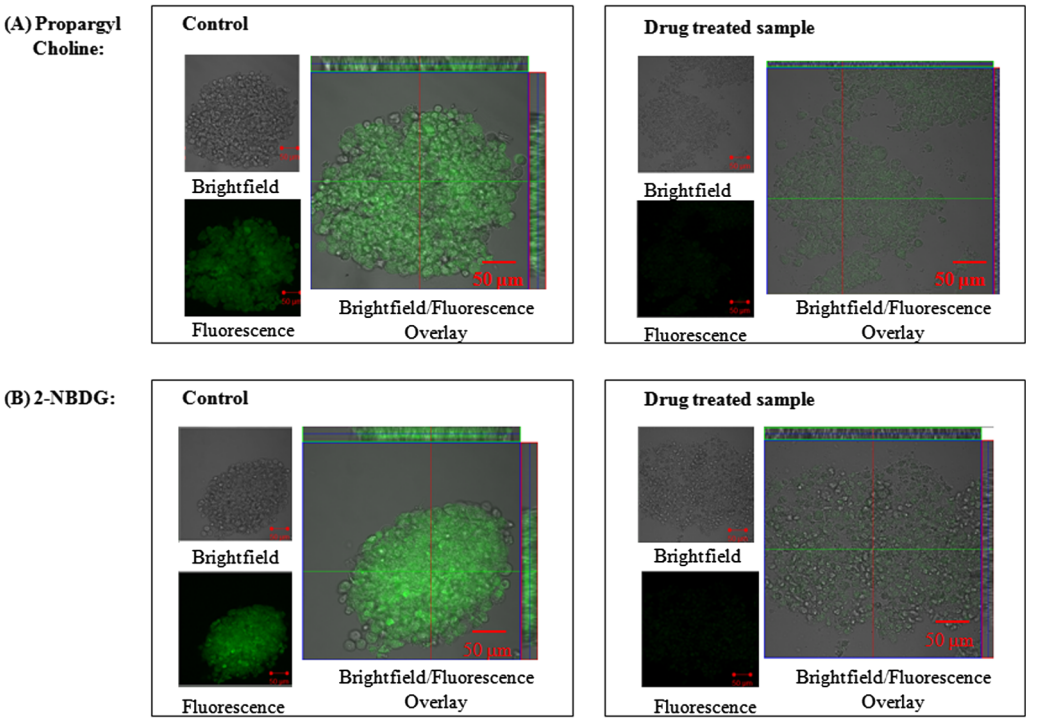

Fig. 6 Confocal fluorescence, brightfield, and brightfield/fluorescence overlay images of multicellular tumor spheroids (MTS) labeled with (a) propargyl choline and (b) 2-NBDG. Control: MTS without cisplatin treatment. Drug treated sample: MTS treated with 100 μM of cisplatin for 48 h.

of this study validate that the click chemistry reactions have high specificity and efficiency in cellular and tissue environments and these results are in agreement with prior studies.[23,27,28]

Glucose is the major source of energy supply and choline is a critical component for membrane synthesis in rapidly dividing and proliferating cancer cells. This study compares cisplatin induced changes in the intracellular uptake of these two distinct but key metabolites (choline and glucose). Treatment of cells and spheroids with cisplatin resulted in a significant reduction in cellular uptake and intracellular retention of both choline and deoxyglucose analogues. The results also show that following cisplatin treatment changes in the uptake of propargyl choline can be detected at an earlier time point as compared to the changes in the uptake of 2-NBDG. Cisplatin induced changes in the uptake and intracellular retention of propargyl choline and 2-NBDG were compared with conventional cell viability and apoptosis assays. This comparison demonstrates higher sensitivity of both 2-NBDG and propargyl choline to detect the response of cancer cells to cisplatin treatment as compared to the conventional viability and apoptosis assays.

One of the key mechanisms for the therapeutic activity of cisplatin is based on the binding of cisplatin to DNA to form cisplatin-DNA adducts (formation of chemical linkage between cisplatin and DNA molecules). The cisplatin DNA adduct formation induces apoptosis in cells. Changes in the uptake and intracellular retention of metabolites prior to cellular apoptosis reflect a range of biochemical changes that can be detected in addition to the formation of cisplatin-DNA adducts. This observation based on the results of this study is also supported by a recent study.[21] In addition to DNA damage, studies have also shown significant early changes in the plasma membrane (membrane fluidity, inhibition of NHE1 exchanger) as a result of cisplatin treatment. These changes in the plasma membrane can trigger the Fas death receptor pathway, leading to apoptosis of cells.[29] Further studies are needed to describe the molecular pathways that mediate the effect of cisplatin on intracellular uptake and retention of choline and glucose analogues. These fundamental studies will complement the development and validation of molecular imaging probes for clinical applications.

To demonstrate the translation of optical molecular imaging probes from a 2-d cell culture model to a 3-d model system, multicellular spheroids of HeLa cells were generated using the experimental approach described in the materials and methods section. Imaging measurements demonstrate that both propargyl choline and 2-NBDG can detect the response of individual cancer cells to cisplatin treatment in 3-d spheroids. 3-d spheroids have been used in cancer research as an effective approach to screen for drugs[24,30,31] and to understand the influence of 3-d architecture on the drug response of cancer cells.[32–35] Developing imaging approaches to noninvasively evaluate the response of 3-d tumor spheroids to therapies can facilitate both the rapid screening of drugs in a laboratory environment as well as pretherapeutic assessment of the response of clinically isolated samples including tumor biopsies.

Current methods to assess the drug response in patients are predominantly limited to post-treatment evaluation of patients.[36] For predicting the drug response prior to cancer therapy, an *ex vivo* histo-culture approach has been proposed.[37,38] In this approach, the tissue biopsies isolated from patients are minced and incubated with target drugs for an extended period of time ranging from a couple of days to over a week. Following the extended incubation of clinical samples with chemotherapy drugs, the response is assessed using the MTT assay. The optical imaging approach developed in this study can be used for rapid assessment of the response of isolated tissues or cells from patients. For *ex vivo* applications, the imaging approach can significantly reduce the incubation time required for evaluating the drug response in clinical samples. This is important as longer incubation time outside the *in vivo* environment can influence cellular physiology of isolated clinical samples and adversely

impact the accuracy of the MTT measurements. Since the optical imaging approach measures the response of individual cells, it can detect a small fraction of resistant cells within a tissue or clinical sample. Further research is required to correlate the changes in the metabolic activity of cells and tissues with effective therapeutic dosage for a given tumor size. Further studies are also needed to evaluate sensitivity and specificity of these imaging methods in detecting cisplatin resistant cells in a heterogeneous cell population. It is envisioned that development of such an approach will enable clinicians to identify tumors with resistance to cisplatin and select alternative therapies that may include combination of cisplatin with other drugs.

In addition to *ex vivo* studies, this approach has a potential for *in vivo* imaging. To detect optical signal in an *in vivo* environment, these imaging probes may be combined with fiber optic needle biopsy systems to detect the optical signal deep within the tumor.[39] For *in vivo* translation of these probes, further research is needed to develop copper free click chemistry that can be specifically used for imaging intracellular targets in living cells within tissues.[40,41]

5 Conclusion

Changes in choline metabolic activity in cancer cells due to cisplatin treatment were specifically detected using optical imaging. Using the optical imaging approach, changes in the uptake of propargyl choline as a function of cisplatin concentration and incubation time were quantified. The comparison between propargyl choline and 2-NBDG demonstrate that the imaging approach based on the choline analogue was more sensitive in detecting the early response of cancer cells to cisplatin treatment. Compared with the conventional cell viability assays (MTT and PI/Annexin V staining), the imaging approaches based on propargyl choline and 2-NBDG had higher sensitivity to evaluate the early response of cancer cells to cisplatin treatment. These results indicate that changes in the metabolic activity of cancer cells precede loss of cell viability. The results of the study also demonstrate the feasibility of imaging drug response in 3-d multicellular spheroids. The potential application of this approach may include optimization of therapeutic dosage and selection of individualized therapies.

Appendix

Supplementary Data: Specificity of uptake of propargyl choline assessed based on the competition assay between propargyl choline and unmodified choline chloride. (a) HeLa cells were incubated with a mixture of a fixed concentration 2 mM of propargyl choline and increasing concentration of unmodified choline (choline chloride) for 1 h. The concentration of choline chloride was varied from 0 to 60 mM. (b) MFI of labeled cells (indicating uptake of propargyl choline) as a function of increasing concentration of choline chloride.

References

1. J. Woodcock, "The prospects for "personalized medicine" in drug development and drug therapy," *Clin. Pharmacol. Ther.* **81**(2), 164–169 (2007).
2. A. A. Neves and K. M. Brindle, "Assessing responses to cancer therapy using molecular imaging," *BBA-Rev. Cancer* **1766**(2), 242–261 (2006).
3. S. Rehman and G. C. Jayson, "Molecular imaging of antiangiogenic agents," *Oncologist* **10**(2), 92–103 (2005).
4. J. Czernin, W. A. Weber, and H. R. Herschman, "Molecular imaging in the development of cancer therapeutics," *Ann. Rev. Med.* **57**, 99–118 (2006).
5. L. Troy et al., "Cisplatin-based therapy: a neurological and neuropsychological review," *Psycho.-Oncol.* **9**(1), 29–39 (2000).
6. I. E. Smith and D. C. Talbot, "Cisplatin and its analogs in the treatment of advanced breast-cancer—a review," *Brit. J. Cancer* **65**(6), 787–793 (1992).
7. R. S. Go and A. A. Adjei, "Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin," *J. Clin. Oncol.* **17**(1), 409–422 (1999).
8. W. G. Mckenna et al., "The use of magnetic-resonance imaging and spectroscopy in the assessment of patients with head and neck and other superficial human malignancies," *Cancer* **64**(10), 2069–2075 (1989).
9. A. R. de Molina et al., "Increased choline kinase activity in human breast carcinomas: clinical evidence for a potential novel antitumor strategy," *Oncogene* **21**(27), 4317–4322 (2002).
10. A. R. de Molina et al., "Overexpression of choline kinase is a frequent feature in human tumor-derived cell lines and in lung, prostate, and colorectal human cancers," *Biochem. Bioph. Res. Co.* **296**(3), 580–583 (2002).
11. J. E. Vance and D. E. Vance, "Phospholipid biosynthesis in mammalian cells," *Biochem. Cell Biol.* **82**(1), 113–128 (2004).
12. I. J. de Jong et al., "C-11-choline positron emission tomography for the evaluation after treatment of localized prostate cancer," *Eur. Urol.* **44**(1), 32–38 (2003).
13. A. J. Breeuwsma et al., "Detection of local, regional, and distant recurrence in patients with PSA relapse after external-beam radiotherapy using (11)C-choline positron emission tomography," *Int. J. Radiat. Oncol.* **77**(1), 160–164 (2010).
14. A. Shukla-Dave et al., "Prediction of treatment response of head and neck cancers with P-31 MR spectroscopy from pretreatment relative phosphomonoester levels," *Acad. Radiol.* **9**(6), 688–694 (2002).
15. D. L. Morse et al., "Response of choline metabolites to docetaxel therapy is quantified *in vivo* by localized (31)P MRS of human breast cancer xenografts and *in vitro* by high-resolution (31)P NMR spectroscopy of cell extracts," *Magn. Reson. Med.* **58**(2), 270–280 (2007).
16. R. Weissleder and U. Mahmood, "Molecular imaging," *Radiology* **219**(2), 316–333 (2001).
17. J. Kurhanewicz et al., "Analysis of cancer metabolism by imaging hyperpolarized nuclei: prospects for translation to clinical research," *Neoplasia* **13**(2), 81–97 (2011).
18. M. Dean, T. Fojo, and S. Bates, "Tumour stem cells and drug resistance," *Nat. Rev. Cancer* **5**(4), 275–284 (2005).
19. G. Kelloff et al., "Progress and promise of FDG-PET imaging for cancer patient management and oncologic drug development," *Clin. Cancer Res.* **11**(8), 2785–2808 (2005).
20. N. Nitin et al., "Molecular imaging of glucose uptake in oral neoplasia following topical application of fluorescently labeled deoxy-glucose," *Int. J. Cancer* **124**(11), 2634–2642 (2009).
21. T. Egawa-Takata et al., "Early reduction of glucose uptake after cisplatin treatment is a marker of cisplatin sensitivity in ovarian cancer," *Cancer Sci.* **101**(10), 2171–2178 (2010).
22. S. R. Millon et al., "Uptake of 2-NBDG as a method to monitor therapy response in breast cancer cell lines," *Breast Cancer Res. Tr.* **126**(1), 55–62 (2011).
23. C. Y. Jao et al., "Metabolic labeling and direct imaging of choline phospholipids *in vivo*," *Proc. Natl. Acad. Sci. USA* **106**(36), 15332–15337 (2009).
24. J. Friedrich et al., "Spheroid-based drug screen: considerations and practical approach," *Nat. Protoc.* **4**(3), 309–324 (2009).
25. M. van Engeland et al., "Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure," *Cytometry* **31**(1), 1–9 (1998).
26. K. Takara et al., "Molecular changes to HeLa cells on continuous exposure to cisplatin or paclitaxel," *Cancer Chemoth. Pharm.* **58**(6), 785–793 (2006).
27. V. V. Rostovtsev et al., "A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes," *Angew. Chem. Int. Ed. Engl.* **41**(14), 2596–2599 (2002).
28. C. W. Tornoe, C. Christensen, and M. Meldal, "Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides," *J. Org. Chem.* **67**(9), 3057–3064 (2002).

29. A. Rebillard, D. Lagadic-Gossmann, and M. T. Dimanche-Boitrel, "Cisplatin cytotoxicity: DNA and plasma membrane targets," *Curr. Med. Chem.* **15**(26), 2656–2663 (2008).
30. L. A. Kunz-Schughart, "Multicellular tumor spheroids: intermediates between monolayer culture and *in vivo* tumor," *Cell Biol. Int.* **23**(3), 157–161 (1999).
31. L. A. Kunz-Schughart et al., "The use of 3-D cultures for high-throughput screening: the multicellular spheroid model," *J. Biomol. Screen.* **9**(4), 273–285 (2004).
32. M. Wartenberg et al., "Doxorubicin distribution in multicellular prostate cancer spheroids evaluated by confocal laser scanning microscopy and the optical probe technique," *Cytometry* **31**(2), 137–145 (1998).
33. B. Weigelt et al., "HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment," *Breast Cancer Res. Tr.* **122**(1), 35–43 (2010).
34. M. Pickl and C. H. Ries, "Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab," *Oncogene* **28**(3), 461–468 (2008).
35. I. Rizvi et al., "Synergistic enhancement of carboplatin efficacy with photodynamic therapy in a three-dimensional model for micrometastatic ovarian cancer," *Cancer Res.* **70**(22), 9319–9328 (2010).
36. P. Therasse et al., "New guidelines to evaluate the response to treatment in solid tumors," *J. Natl. Cancer I.* **92**(3), 205–216 (2000).
37. I. S. Horn et al., "Heterogeneity of epithelial and stromal cells of head and neck squamous cell carcinomas in *ex vivo* chemoresponse," *Cancer Chemoth. Pharm.* **65**(6), 1153–1163 (2010).
38. F. Pirnia et al., "*Ex vivo* assessment of chemotherapy-induced apoptosis and associated molecular changes in patient tumor samples," *Anticancer Res.* **26**(3A), 1765–1772 (2006).
39. B. A. Flusberg et al., "Fiber-optic fluorescence imaging," *Nat. Methods* **2**(12), 941–950 (2005).
40. J. M. Baskin et al., "Copper-free click chemistry for dynamic *in vivo* imaging," *P. Natl. Acad. Sci. USA* **104**(43), 16793–16797 (2007).
41. P. V. Chang et al., "Copper-free click chemistry in living animals," *P. Natl. Acad. Sci. USA* **107**(5), 1821–1826 (2010).