Published in final edited form as:

J Control Release. 2010 March 19; 142(3): 457–464. doi:10.1016/j.jconrel.2009.10.034.

Non-Invasive Monitoring of Intra-Tumor Drug Concentration and Therapeutic Response using Optical Spectroscopy

Gregory M. Palmer, PhD¹, Richard J. Boruta¹, Benjamin L. Viglianti, MD, PhD⁵, Lan Lan, PhD³, Ivan Spasojevic, PhD⁴, and Mark W. Dewhirst, DVM, PhD^{1,2}

¹Department of Radiation Oncology, Duke University Medical Center

²Department of Biomedical Engineering, Duke University Medical Center

³Department of Biostatistics and Bioinformatics, Duke University Medical Center

⁴Department of Medicine Duke University Medical Center

⁵Department of Radiology, University of Michigan Ann Arbor MI 48103

Abstract

Optical spectroscopy was used to monitor changes in tumor physiology with therapy, and its influence on drug delivery and treatment efficacy for hyperthermia treatment combined with free doxorubicin or a low-temperature sensitive liposomal formulation. Monte Carlo-based modeling techniques were used to characterize the intrinsic absorption, scattering, and fluorescence properties of tissue. Fluorescence assessment of drug concentration was validated against HPLC and found to be significantly linearly correlated ($r=0.88$). Cluster analysis on the physiologic data obtained by optical spectroscopy revealed two physiologic phenotypes prior to treatment. One of these was relatively hypoxic, with relatively low total hemoglobin content. This hypoxic group was found to have a significantly shorter time to reach 3 times pre-treatment volume, indicating a more treatment resistant phenotype ($p=0.003$). Influence of tumor physiology was assessed in more detail for the liposomal doxorubicin + hyperthermia group, which demonstrated a highly significant correlation between pre-treatment hemoglobin saturation and tumor growth delay, and also between post-hyperthermia total hemoglobin content and tumor drug delivery. Finally, it was found that the doxorubicin concentration, measured *in vivo* using fluorescence techniques significantly predicted for chemoresponse (hazard ratio: 0.34, $p=0.0007$). The ability to characterize drug delivery and tumor physiology *in vivo* makes this a potentially useful tool for evaluating the efficacy of targeted delivery systems in preclinical studies, and may be translatable for monitoring and predicting individual treatment responses in the clinic.

Keywords

Drug delivery; liposome; doxorubicin; pharmacokinetics; fluorescence spectroscopy

© 2009 Elsevier B.V. All rights reserved.

Corresponding Author: Gregory Palmer, phone: 919-613-5053, fax: 919-684-8718, greg.palmer@duke.edu Address: Duke University Medical Center Dept. Radiation Oncology Box 3455 Durham, NC 27710.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

This study was motivated by the clinical and pre-clinical need for a cost-effective, quantitative means of monitoring drug delivery kinetics and the influence of tumor physiology therapeutic response. Optical spectroscopy techniques are sensitive to the wide range of important biological molecules and processes that affect the absorption, scattering, and fluorescence properties of the tissue, and can potentially report on important aspects of chemoresponse. Notably, absorption in the visible wavelength range is dominated by hemoglobin, which allows for characterization of tissue blood volume and hemoglobin oxygen saturation. Scattering is affected by local inhomogeneities in the refractive index, such as in cellular organelles, and the extracellular matrix. Cells also contain sources of intrinsic fluorescence, which include the electron carriers NADH and FAD, indicators of tissue metabolic function [1,2]. Since this method is non-invasive and rapid, it can be used to assess tissue optical properties in tissue over multiple time points. Serial measurements can assess tissue response to treatment (pharmacodynamics), and for the case of fluorescent drugs, measurement of drug uptake (pharmacokinetics) is also possible.

The emergence of nanoparticles for use as drug carriers also presents an emerging opportunity for optical techniques to characterize drug delivery [3]. The ability to create multi-functional nanoparticles that may be conjugated with a fluorophore [4] enables a dynamic measurement of drug carrier kinetics into the tumor tissue *in vivo*. In addition, because many nanoparticle delivery systems rely on passive enhance permeability and retention effect (EPR) [5], a non-invasive means of assessing vascular function may be useful in predicting the accumulation of nanoparticles into a given tumor, and could guide the selection of delivery mechanism to suit the pathophysiology of an individual tumor.

Recent clinical and preclinical research has focused on developing optical techniques to monitor and predict therapeutic response *in vivo*, with several pilot and case studies reporting changes in physiologic parameters upon treatment [6-8]. Notably, Cerussi et al. found that early (1 week) drops in total hemoglobin and water content were related to response to neoadjuvant chemotherapy of breast cancer with doxorubicin [9]. This method was able to discriminate pathologic responders from non-responders with 100% accuracy in a small sample of 11 patients. In a preclinical study, Vishwanath et al. investigated changes in optical parameters upon treatment of the 4T1 mammary carcinoma cell line with maximum tolerated dose (MTD) doxorubicin [10]. They showed that the scattering coefficient had significant correlation with the necrotic fraction, and that tumors treated with doxorubicin showed significant increases in hemoglobin saturation at day 10, relative to controls. Doxorubicin fluorescence has been used to characterize drug delivery and distribution in cells, animal tumors, and clinical samples [11-14], but has not previously been used to characterize drug accumulation or pharmacokinetics *in vivo*, to our knowledge.

This study seeks to explore the potential role of optical spectroscopy in monitoring and predicting therapeutic response in a preclinical model. Physiologic changes induced by hyperthermia, and treatment with doxorubicin encapsulated in low-temperature sensitive liposome (LTSL) nanoparticles were evaluated. We have previously shown that hyperthermia effects tissue perfusion and oxygenation [15], whereas LTSL encapsulated doxorubicin + hyperthermia (HT) induces vascular shutdown [16]. Each of these methods could produce optical effects, but it is not clear what effect predominates when LTSL-doxorubicin is combined with hyperthermia. Since doxorubicin is also fluorescent [17], we also quantified drug accumulation by performing fluorescence spectroscopy.

Methods

Animal Treatment and Tumor Growth Delay Study

Dual flank tumors were grown in nude mice, by injecting 3 million SKOV-3 ovarian cancer cells subcutaneously. Tumor sizes were measured every three days throughout the study using calipers, using the equation $V = \pi L W^2 / 6$, where V is the volume, L is the longest dimension of the tumor, and W is the width of the tumor at a right angle to L . Animals were monitored until one of tumors grew to a volume of 150–200 mm³. Treatment was given if the opposing tumor was measureable. The animals were then randomized to one of 6 treatment groups, saline (control), free doxorubicin, and LTSL-encapsulated doxorubicin (LTSL-doxorubicin), all with and without HT. Drug formulations were prepared using established protocols [18]. The LTSL components were obtained from the commercial Thermodox formulation (Celsion, Inc.). This consists of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene glycol 2000 (DSPE-PEG-2000) in the molar ratio of 90:10:4. For the animals receiving hyperthermia, pentobarbital was used to anesthetize the animal, with 85 mg/kg injected i.p. For all other animals, and for later optical measurements, 1.5% isoflurane was used for anesthesia. In the case of free doxorubicin or LTSL-dox, the concentration of drug was 2 mg/ml, and was delivered via tail vein injection for a dose of 5 mg/kg. Saline was injected at the same volume for the control groups. Hyperthermia treatment was administered by placing the leg of the larger tumor into a water bath heated to 43.5 °C for 1 hour (this yields an intratumoral temperature of approximately 42°C). A plastic bag was draped over the mouse to keep the animal dry during this procedure. All procedures were approved by Duke University's Institutional Animal Care and Use Committee.

Measurement of Doxorubicin Concentration

A second cohort of animals was utilized to characterize the concentration of drug accumulating within the tumor, for comparison to optical data. In this study, animals were treated as above, with $n=8$ animals per group, excluding the control (saline) groups. All animals received pentobarbital anesthesia (85 mg/kg, injected i.p.). After treatment and optical measurements, the animals were immediately sacrificed and tumors excised and flash frozen in liquid nitrogen. Drug concentration was then quantified using high performance liquid chromatography (HPLC).

HPLC Measurements

Whole frozen tumors were pulverized by cryo-crushing under liquid nitrogen. The powder was weighed and further homogenized by stator (polytetrafluoroethylene) – rotor (glass) homogenizer after adding appropriate amount of deionized water (3 mL per gram tissue). The homogenate was stored at –80°C as 100 µL aliquots in 2 µL polypropylene (PP) screw-cap vials. On the day of analysis, 100 µL aliquot of homogenate was thawed at room temperature and 100 µL of 2.5 mg/µL daunorubicin (internal standard, Sigma-Aldrich) in water was added. The solution was then vortex-mixed for 5 s, and left to equilibrate at room temperature for 10 min. Liquid-liquid extraction was accomplished by adding 1.5 µL of chloroform/isopropanol (4/1) and 25 µL of 6% sodium tetraborate (Sigma-Aldrich) in water, vigorous agitation (Fast-Prep FP150, Thermo-Savant, speed 4, 2 × 45 s), and centrifugation (16,000 g, 3 min, 4 °C). The upper layer was discarded, 1 µL of lower (organic) layer transferred to a labeled 2 µL PP vial, and solvents removed by gentle stream of nitrogen at room temperature. The dry residue was reconstituted by adding first 50 µL of methanol followed by vortex-mixing and addition of 250 µL of mobile phase A (see below). The solution was agitated (Fast-Prep, speed 4, 45 s) and centrifuged (16,000 g, 3 min, 4 °C). The supernatant was removed into all-plastic polypropylene syringe equipped with 22G 1.5” needle and filtered through a 0.45 µm nylon filter (National Scientific Co.) into a polypropylene autosampler vial (~150 µL). HPLC-

fluorescence equipment: Waters 2695 HPLC module (degasser, pump, autosampler, column oven) and Waters 2475 fluorescence detector. Column: Agilent Eclipse XDB, C₈, 4.6 × 150 mm. Guard column: Phenomenex, C₈, 3 × 4 mm. Mobile phase A: 0.5% H₃PO₄ (85%, Fisher Scientific), 0.5% tetrahydrofuran (J.T. Baker), 5% acetonitrile (EMD Chemicals). Mobile phase B: 50% acetonitrile, 50% methanol (EMD Chemicals). Gradient elution profile: 0-12 min 95-55% A, 12-13 min 55-10% A, 13-14 min 10% A, 14-15 min 10-95% A. Flow rate: 1.5 µL/min. Column oven temperature: 50 °C. Autosampler temperature: 4 °C. Injection volume: 10 µL. Detector settings: 480 nm (excitation)/500 nm (emission), gain 10. Run time of analysis per sample: 30 min. The analysis of each sample set was accompanied by the analysis of the calibration standard. Samples were prepared by using non-treated tumor tissue which was homogenized by the same procedure as described above and spiked with 0.000, 0.016, 0.08, 0.4, 2, and 10 µg/µL doxorubicin (Sigma-Aldrich). Empower (Waters) software was utilized for linear regression fitting to calibration data with weighing factor 1/x. Accuracy acceptance criterion was 85% at all concentration levels, except for the lowest limit of quantification (16 ng/µL) for which it was set to 80%.

Optical Measurements

The measurement method consists of illuminating tissue with specific wavelength(s) of light, and recording the exiting light intensity as a function of wavelength. Two types of measurements are obtained: diffuse reflectance and fluorescence spectroscopy. With diffuse reflectance the tissue is illuminated with light, and the light exiting the tissue at the same wavelength is monitored. This technique is sensitive to the absorption and scattering properties of the tissue. Fluorescence spectroscopy involves illuminating the tissue at a given wavelength and recording at longer wavelengths. This is sensitive to the absorption, scattering, and fluorescence properties at the excitation and emission wavelengths. The combination of the two techniques permits multiparametric characterization of the tissue's optical properties.

Because of the complex interaction of tissue optical processes, it is necessary to employ light transport models to determine the underlying absorption, scattering, and fluorescence properties that influence the measured spectra. Several groups have developed such techniques including empirical relationships, diffusion-based approximations to light transport, and Monte Carlo models of light transport. We have previously developed and validated a Monte Carlo based model of light-tissue interaction that enables the quantitative extraction of absorber concentrations, scatterer properties, and intrinsic fluorescence properties from measured optical spectra; these methods are employed in this study [19-21]. The primary advantage of our approach over existing commercial offerings is the ability to quantify the total hemoglobin, hemoglobin saturation, as well as fluorophore concentration using the modeling techniques described. Most commercial offerings characterize fluorescence intensity, but do not enable quantification of absolute concentration and none to our knowledge enable quantification of tissue absorption, scattering and fluorescence properties in a combined instrument.

Optical measurements were performed by placing a fiber optic probe on the surface of the tumor; such that the probe was flush with the tumor surface. The probe consisted of a set of 40 illumination fibers, arranged in a 4×10 column, separated from a second set of 4×10 collection fibers by a 2 mm glass spacer. The spacer was composed of a dark neutral density filter to block scattered light. This served the dual purpose of providing a large fixed separation to increase probing depth, thus minimizing the effects of skin, while also approximating the refractive index of the silica fibers, thus simplifying the modeling by presenting a uniform optical interface to the tissue surface.

The fiber optic probe was coupled to a Skinskan fluorometer (HORIBA Jobin Yvon). This instrument consists of a 150 W xenon arc lamp, dual scanning monochromators, and a photomultiplier tube detector. This instrument has a fixed 5 nm bandwidth. Four scans were

performed for each measurement: 1) a diffuse reflectance synchronous scan from 350-700 nm, 2) a fluorescence emission spectrum at 350 nm excitation, 375-555 nm emission, 3) a fluorescence emission spectrum at 460 nm excitation, 485-645 nm emission, and 4) a fluorescence emission spectrum at 480 nm excitation, 500-680 nm emission. All spectra were acquired with a 5 nm increment. These wavelengths were chosen to allow for characterization of the hemoglobin content and saturation, scattering properties, and NADH, FAD, and doxorubicin content of the tissue. The high voltage of the PMT was set to 600 V for the diffuse reflectance and 950 V for the fluorescence measurements. Optical measurements were made at baseline (just prior to treatment), immediately post treatment (~65 minutes post injection, immediately after completion of hyperthermia treatment), and every three days subsequently, up to 15 days post-treatment.

Reflectance measurements were calibrated daily by normalization to a 99% reflectance puck measurement (Labsphere, Inc.), measured at least twice daily. Fluorescence measurements were calibrated by normalizing to a rhodamine standard, also measured at least twice daily. Dark signal was determined by measurement in water, and was found to be negligible.

Optical Modeling

Modeling was performed using previously described Monte Carlo models of light transport [19,21], which have been extensively validated [19,21,22] and applied to a number of tissue studies [20,23-27]. This consists of a two step analysis whereby the absorption and scattering properties are first extracted from the diffuse reflectance spectrum [19]. Then the intrinsic fluorescence (corrected for the effects of absorption and scattering) is then determined by applying a correction factor to the measured fluorescence spectra, based on these optical properties [21]. A brief description of these models follows.

The diffuse reflectance models the effects of the absorption and scattering properties on the measured diffuse reflectance spectrum. In order to limit the number of free parameters, scattering was approximated using a power law relationship, given by $\mu_s' = A(\lambda/400)^{-B}$, where μ_s' is the reduced scattering coefficient, λ is the wavelength in nm, A is the scattering amplitude, and B is the scatter power. This relationship has previously been shown to well approximate tissue scattering properties for a variety of tissue types [6,7,28]. Absorption was modeled using the spectra of oxygenated and deoxygenated hemoglobin [29]. A baseline tissue absorption term corresponding to rat skin tissue absorption in the absence of hemoglobin and melanin was also included to account for other sources of absorption present in tissue (which likely incorporates a variety of chromophores such as carotenoids) [30]. This term is defined by the empirical equation $\mu_a = c * (0.244 + 85.3 * e^{-(\lambda - 154)/66.2})$, where μ_a is the absorption coefficient, λ is the wavelength in nm, and c is the free parameter determined in the fitting algorithm. In addition, a correction factor accounting for the effect of chromophore packing into the blood vessels on the apparent absorption spectrum was included [31]. The extraction of the optical properties is an iterative process whereby the purported absorber concentrations, vessel size parameter, and scattering amplitude and power are varied such that the simulated spectrum based on these optical properties most closely matches the measured spectrum, using a least squares fitting algorithm. A reference phantom consisting of polystyrene spheres and hemoglobin is used to calibrate for system throughput and wavelength response.

The effects of absorption and scattering at the excitation and emission wavelengths can then accounted for in the fluorescence spectra, to extract a parameter proportional to the product of the fluorophore concentration, quantum yield, and wavelength-dependent absorption and emission properties [21]. For a stable fluorophore, this would be proportional to changes in concentration only. For these data, diffuse reflectance fits were obtained over the range of 460-610 nm. This allowed for extraction of hemoglobin oxygen saturation (oxygenated hemoglobin / total hemoglobin), total hemoglobin content (related to blood volume), the

scattering properties as a function of wavelength, and also enabled extraction of intrinsic fluorescence at 460 and 480 nm excitation. Because reflectance fits were not carried out down to 350 nm, raw fluorescence intensities only were utilized for 350 nm emission spectra. This was done to enable simplification of the reflectance model to avoid additional UV absorbers. It also allowed for deeper mean penetration into the tumor, by removing the highly absorbing shorter wavelengths.

Statistical Analysis

Once the intrinsic fluorescence spectra were extracted, the ability to extract the doxorubicin concentration based on this was tested. Because the doxorubicin fluorescence was of the same magnitude as the tissue autofluorescence, some method was needed to correct for this. A variety of methods were tested, including multivariate curve resolution [32] and simple wavelength ratios, with the one showing the best linear correlation with HPLC chosen for subsequent analysis.

Having extracted all of these optical parameters, it was then hypothesized that by characterizing tissue oxygenation and metabolic activity, it would be possible to identify tumors that would be more or less responsive to treatment. This is supported by existing data demonstrating that hypoxic tumors exhibit more aggressive, treatment resistant phenotypes [33]. A k-means cluster analysis [34] was carried out based on the baseline (pre-treatment) scan to see if different physiologic subgroups with different treatment responses would emerge. The k-means analysis was then performed using a squared-Euclidean distance function. K-means analysis is an iterative technique, whereby the group members and their respective group centroids are optimized such that the distance from all points to their respective group centroid is minimized. This results in a set of clusters that are as compact and well-separated as possible. The number of distinct clusters was determined empirically by repeating the analysis with 2-5 clusters, and determining the number of clusters that maximized the average distance from a given point in one cluster, to all points in the other clusters.

Finally, in order to ascertain the ability of optical parameters to predict for treatment outcome, a Cox proportional hazard model was fit to the data to model the relationship of the time to reach 3 times pre-treatment volume to some covariates simultaneously. The covariates include the initial (pre-treatment) and 1 hour (post-treatment) hemoglobin saturation and total hemoglobin parameters, and drug uptake assessed by fluorescence post-treatment. These early response parameters were chosen to examine the ability of early changes to predict for long term outcome.

Results

The optical protocol was an add-on to a study investigating the efficacy of LTSL-doxorubicin +HT on primary and distant unheated tumor sites, the results of which are in preparation to be published elsewhere. These optical measurements were made on the primary site. The pertinent finding of the growth delay portion of the study are that LTSLdoxorubicin+HT showed significantly longer growth delay using log-rank test ($p < 0.0001$). The remaining groups did not show significant efficacy, relative to control.

Figure 1 shows representative diffuse reflectance and fluorescence spectra, and modeled data for a LTSL-dox treated animal. For diffuse reflectance spectra, the modeled data are the result of a least squared fit used to extract the tissue absorption and scattering properties and so closely match one another. For the fluorescence spectra, the modeled data are corrected for the influence of absorption and scattering and so would not be expected to overlap for a turbid medium. The intrinsic fluorescence spectra in Figure 1(b) were not calculated because the reflectance fitting range did not extend into the UV wavelength at which this spectra was

excited (350 nm) - this simplified the modeling by eliminating the need for potential UV absorbers. Fluorescence spectra in Figure 1(c) are normalized to mean baseline values to appreciate the effects of absorption and scattering on the line shape of the measured spectra. It can be seen that the diffuse reflectance intensity decreases after treatment, which is indicative of an increase in absorption due to a higher blood volume. It can furthermore be seen that fluorescence emission at wavelengths greater than 550 nm is higher in intensity after treatment. The autofluorescence present pre-treatment is likely attributed to flavin fluorescence, which has an emission maximum at 535 nm [35]. After treatment, the peak emission wavelength shifts to the red and increases in intensity, which indicates the presence of doxorubicin, which has an emission maximum at 593 nm [17]. The measured (raw) fluorescence spectrum is also shown as the solid line. This can be seen to be significantly distorted by the effects of absorption and scattering, which would not allow this spectrum to be characterized by a linear combination of contributing fluorophores, due to the confounding effects of turbidity. Correction for absorption and scattering restores the peak emission wavelength to approximately 590 nm, as would be expected for doxorubicin fluorescence (compare solid and dashed lines in Fig. 1(c)).

Figure 2 shows the correlation between the doxorubicin concentration determined by HPLC, and the ratio of average fluorescence acquired at 580-610 nm (doxorubicin peak) to that of 510-530 nm (flavin peak) emission, at 480 nm excitation. This ratio was chosen because it was found to accurately correct for the influence of tissue autofluorescence and provided a strong linear correlation between HPLC and tissue fluorescence measurements (Pearson's correlation: $r=0.88$, $p=7e-21$). This will be referred to as the doxorubicin fluorescence ratio. Also, for this portion of the study, both the primary and secondary tumors were measured. As expected, there is higher drug accumulation for the primary (heated) tumor, which is driven primarily by the targeted accumulation of drug in the LTSL-doxorubicin+HT group. A linear least squares trend line was fit to these data to allow for calibration of the doxorubicin fluorescence ratio to units of absolute concentration.

In the primary study, two different types of anesthesia were used. During hyperthermia it was necessary to have a prolonged period of anesthesia, and as a result, pentobarbital (85 mg/kg, intraperitoneal injection) was used for the baseline and 1 hour post-treatment measurements. For all other measurements, 1.5-2.5% isoflurane in oxygen, was used due to its fast acting nature, and the ability to use it serially. In order to assess the potential influence of this, the group means of the animals receiving hyperthermia were compared to those not receiving hyperthermia at the pretreatment baseline measurement, since these animals were otherwise identical at that point. It was found that there was not a significant difference for either total hemoglobin content or hemoglobin saturation by Wilcoxon rank-sum test.

Figure 3 shows a summary of the more interesting findings from the optical data. The plot is divided into three subplots, each corresponding to a different optical parameter, namely (a) doxorubicin concentration (measured optically), (b) total hemoglobin, and (c) hemoglobin saturation. The doxorubicin concentration was calibrated to the linear regression line shown in Figure 2, and the baseline (pre-treatment) value was subtracted to account for inter-animal variability in autofluorescence properties. Within each subfigure, the six treatment groups are plotted at each of the seven time points used in this study using box-plots. Notable features to point out include the fact that the doxorubicin concentration is significantly higher in the LSTL-Doxorubicin+HT group at the 1 hour time point, relative to the other groups receiving doxorubicin ($p=2e-4$, by Wilcoxon rank-sum test). There is a 15 fold increase in doxorubicin concentration in the group median value of LSTL-doxorubicin+HT, relative to free doxorubicin with no HT. There is a 12 fold difference in group means. Groups receiving hyperthermia show an immediate increase in total Hb at the one hour time point ($p=0.007$, Wilcoxon sign-rank test). Following this, the LTSL-doxorubicin+HT group is unique in that the total Hb drops significantly for subsequent time points, compared to baseline ($p=0.02$,

testing mean total Hb on days 6-15, compared to baseline measurement for each animal using Wilcoxon sign-rank test). Finally, the hemoglobin saturation data suggests hyperthermia alone induces increased oxygenation immediately after treatment ($p=0.002$). This effect is not seen however, with the combined therapies.

Next, the ability of optical spectroscopy to identify different tumor phenotypes based on the available physiologic data was explored. This was done using a k-means cluster analysis, whereby the natural clustering of the data was examined on the baseline data (pre-treatment), without making any a priori assumptions about how the data should be clustered, or what the group means should be. It was found that the data could be described adequately by two cluster centroids. Furthermore, these clusters corresponded to the more or less hypoxic phenotypes, namely physiologic cluster 1 exhibited significantly lower hemoglobin saturation (11 ± 7 vs. $28\pm8\%$) and lower total hemoglobin (31 ± 10 vs. $59\pm23\ \mu\text{M}$) (see Figure 4(a)). The average time to reach 3 times pre-treatment volume was then assessed for each group, and it was found that there was a significant difference, and the more hypoxic group (physiologic cluster 1) had a significantly shorter time to reach 3 times pre-treatment volume ($p=0.003$ by log-rank test), indicating these tumors grew significantly faster than the less hypoxic tumors (Figure 4(b-c)). Investigating the influence of tumor physiology on treatment efficacy in more detail yielded two highly significant correlations. Figure 4(d) shows the correlation between the pre-treatment hemoglobin saturation and time to reach 3 times pre-treatment volume ($r=0.8$, $p=0.01$ by Spearman Rank correlation). Figure 4(e) shows the correlation between doxorubicin concentration and the post-treatment total hemoglobin content ($r=0.89$, $p=0.001$ by Spearman rank correlation).

Finally, a Cox proportional hazard model was fit to these data to identify parameters associated with an increased risk of reaching 3 times pre-treatment volume. Five input parameters were chosen, baseline and immediate post treatment measures of hemoglobin saturation and total hemoglobin, as well as the doxorubicin concentration at the one hour time point, assessed optically. Table 1 summarizes the estimates of the hazard ratios. Median values of each parameter were used as the cutoff point to stratify the samples into two groups for each variable. The adjusted hazard of reaching 3 times pre-treatment volume for animals with higher doxorubicin concentration is significantly lower than that for animals with lower doxorubicin concentration (hazard ratio: 0.34, 95% CI, 0.19-0.64, $p=0.0007$). In addition, the hazard of the tumor to reach 3 times pre-treatment volume for animals with higher total hemoglobin is less than that for the animals with lower total hemoglobin pre and post treatment.

Discussion

The major findings of this project include the fact that optical spectroscopy is able to identify expected physiologic changes upon therapy, *in vivo*. This includes increased perfusion and oxygenation after hyperthermia [15], as indicated by increased total hemoglobin content, and hemoglobin saturation, respectively. Also, the reduction in total hemoglobin content at later time points for the LTSL-doxorubicin+HT group (6+ days) could be indicative of the vascular shutdown and regression that has been reported in window chamber models [16]. It was also found that LTSL-doxorubicin+HT showed significantly greater drug accumulation, compared to the other groups, which is also expected based on existing data. Namely, Kong et al. found >25 fold increase in drug accumulation, compared to free doxorubicin, as assessed by HPLC, in testing these liposomes in a xenograft tumor model [36]. In a follow up study, Ponce et al. found an approximately 7 fold increase in mean concentration in a rat fibrosarcoma model for the same treatments [37]. These results are in line with the increase of 15 fold in the group medians, 12 fold in the group means, from the baseline subtracted free doxorubicin (no HT), and LTSLdoxorubicin+HT groups at the 1 hour time point. Potential sources of error include the fact that the change in intrinsic (modeled) doxorubicin fluorescence is linear with

concentration only when the quantum efficiency is unchanged, which may not be the case depending on the microenvironment of the drug and its binding state. However, in the direct comparison with HPLC, a strong linear correlation was noted, indicating that these effects may be minimal ($r=0.88$).

As mentioned in the Introduction, optical techniques have not been used to assess doxorubicin concentration *in vivo*. This is likely due to the difficulties in separating the effects of absorption, scattering, and autofluorescence, for which modeling techniques are required, as seen in Figure 1. The rapid acquisition time of optical techniques (potentially sub-second resolution) could provide critical insight into *in vivo* pharmacokinetics, and provide real-time feedback of drug delivery into a tumor. It is fortuitous that doxorubicin is intrinsically fluorescent, but this approach could be extended to other drugs by using a fluorescently labeled drug carrier, or tagging a fluorophore to a macromolecular therapeutic such as monoclonal antibodies (e.g. herceptin). The physiologic information is all obtained without the use of contrast agents, and so could be directly applied for any treatment type. This type of information could be of particular importance in triggered delivery systems such as the low-temperature sensitive liposomes, to ensure that the expected rapid release of drug is occurring.

The additional parameters available to optical spectroscopy were also found to be relevant to predicting tumor chemoresponse. In particular, cluster analysis revealed two distinct phenotypes, one being more hypoxic and aggressive, and the other being less hypoxic, and having a slower growth rate. This is supported by existing literature, which has shown hypoxic tumors to be more aggressive, metastatic, and treatment resistant than more well-oxygenated tumors [33]. In examining the LTSL-doxorubicin+HT group in more detail, it was found that there was a significant correlation between the pre-treatment hemoglobin saturation and the time to reach 3X treatment volume, which is in agreement with the overall trends. In addition, it was found that there was a highly significant correlation between the doxorubicin concentration and the post-treatment total hemoglobin content. This may be due to the mechanism of drug release in the LTSL formulation, whereby the liposomes rapidly release their drug intravascularly upon exposure to temperatures induced by mild hyperthermia [16]. This concentration gradient drives drug delivery into the tumor. Tumors with a larger blood volume may receive more drug due to this mechanism. This work highlights the importance of non-invasive monitoring of tumor pathophysiology as a biomarker to determine the potential aggressiveness of the tumor. In addition, monitoring of vascular function/morphology may provide insight into the efficacy of a given targeted delivery system for an individual tumor and guide selection of an optimal treatment strategy. Our ultimate objective is to utilize multivariate analysis to predict the treatment response of an individual, based on early changes in physiology and drug pharmacokinetics.

In conclusion, optical spectroscopy was shown to be a useful tool in characterizing drug delivery and physiologic changes induced by therapy. Because it is relatively cheap, fast, and provides quantitative physiologic data, it could prove to be a useful tool for monitoring and predicting therapeutic response. These features also make this technology amenable to clinical translation for many disease sites. The primary challenge is the penetration depth of optical wavelengths, typically on the order of millimeters. We are implementing this approach for monitoring treatment of chest wall recurrence of breast cancer, which provides a superficial location that is ideal for optical measurements. Deeper tissue sites, such as intact breast can be assessed using near infrared wavelengths [6,9], or a catheter-based system.

Acknowledgments

This work was supported by the Department of Defense Breast Cancer Research Program [grant number W81XWH-07-1-0355]; and the National Institutes of Health [grant number 5P01CA042745-22]. The authors would

also like to acknowledge Dr. Nirmala Ramanujam for helpful discussions and generous use of equipment, and Jing Guo who assisted with data analysis.

References

1. Chance B, Schoener B, Oshino R, Itshak F, Nakase Y. Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals. *J Biol Chem* 1979;254(11):4764–4771. [PubMed: 220260]
2. Chance B, Nioka S, Warren W, Yurtsever G. Mitochondrial NADH as the bellwether of tissue O₂ delivery. *Adv Exp Med Biol* 2005;566:231–242. [PubMed: 16594157]
3. Cho KJ, Wang X, Nie SM, Chen Z, Shin DM. Therapeutic nanoparticles for drug delivery in cancer. *Clinical Cancer Research* 2008;14(5):1310–1316. [PubMed: 18316549]
4. Gindy ME, Prud'homme RK. Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. *Expert Opinion on Drug Delivery* 2009;6(8):865–878. [PubMed: 19637974]
5. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *Journal of Controlled Release* 2000;65(12):271–284. [PubMed: 10699287]
6. Choe R, Corlu A, Lee K, Durduran T, Konecky SD, Grosicka-Koptyra M, Arridge SR, Czerniecki BJ, Fraker DL, DeMichele A, Chance B, Rosen MA, Yodh AG. Diffuse optical tomography of breast cancer during neoadjuvant chemotherapy: a case study with comparison to MRI. *Med Phys* 2005;32(4):1128–1139. [PubMed: 15895597]
7. Sunar U, Quon H, Durduran T, Zhang J, Du J, Zhou C, Yu G, Choe R, Kilger A, Lustig R, Loevner L, Nioka S, Chance B, Yodh AG. Noninvasive diffuse optical measurement of blood flow and blood oxygenation for monitoring radiation therapy in patients with head and neck tumors: a pilot study. *Journal of Biomedical Optics* 2006;11(6):064021–064013. [PubMed: 17212544]
8. Bigio IJ, Bown SG. Spectroscopic sensing of cancer and cancer therapy: current status of translational research. *Cancer Biol Ther* 2004;3(3):259–267. [PubMed: 15107613]
9. Cerussi A, Hsiang D, Shah N, Mehta R, Durkin A, Butler J, Tromberg BJ. Predicting response to breast cancer neoadjuvant chemotherapy using diffuse optical spectroscopy. *Proc Natl Acad Sci U S A* 2007;104(10):4014–4019. [PubMed: 17360469]
10. Vishwanath, K.; Yuan, H.; Moore, L.; Bender, J.; Dewhirst, M.; Ramanujam, N. *Biomedical Optics/ Digital Holography and Three-Dimensional Imaging/ Laser Applications to Chemical, Security and Environmental Analysis on CD-ROM*. The Optical Society of America; Washington DC., St. Petersburg, FL: 2008. Longitudinal monitoring of 4T1-tumor physiology in vivo with Doxorubicin treatment via diffuse optical spectroscopy. p. presentation number BTuC3
11. Lankelma J, Dekker H, Fernandez Luque R, Luykx S, Hoekman K, Valk P.v.d. van Diest PJ, Pinedo HM. Doxorubicin Gradients in Human Breast Cancer. *Clin Cancer Res* 1999;5(7):1703–1707. [PubMed: 10430072]
12. Zvi Symon AP, Tzemach Dinah, Lyass Olga, Sucher Erwin, Shezen Elias, Gabizon Alberto. Selective delivery of doxorubicin to patients with breast carcinoma metastases by stealth liposomes. *Cancer* 1999;86(1):72–78. [PubMed: 10391566]
13. Hideki Kawai YM, Kitamura Michihiko, Matsuzaki Ikuo, Hashimoto Masaji, Suzuki Hiroyuki, Abo Shichisaburo. Direct measurement of doxorubicin concentration in the intact, living single cancer cell during hyperthermia. *Cancer* 1997;79(2):214–219. [PubMed: 9010093]
14. Zheng J, Chen C-T, Au J, Wientjes M. Time- and concentration-dependent penetration of doxorubicin in prostate tumors. *The AAPS Journal* 2001;3(2):69–77.
15. Dewhirst MW, Viglianti BL, Lora-Michiels M, Hanson M, Hoopes PJ. Basic principles of thermal dosimetry and thermal thresholds for tissue damage from hyperthermia. *Int J Hyperthermia* 2003;19(3):267–294. [PubMed: 12745972]
16. Chen Q, Tong S, Dewhirst MW, Yuan F. Targeting tumor microvessels using doxorubicin encapsulated in a novel thermosensitive liposome. *Mol Cancer Ther* 2004;3(10):1311–1317. [PubMed: 15486198]
17. Karukstis KK, Thompson EH, Whiles JA, Rosenfeld RJ. Deciphering the fluorescence signature of daunomycin and doxorubicin. *Biophys Chem* 1998;73(3):249–263. [PubMed: 9700924]

18. Kong G, Anyarambhatla G, Petros WP, Braun RD, Colvin OM, Needham D, Dewhirst MW. Efficacy of liposomes and hyperthermia in a human tumor xenograft model: importance of triggered drug release. *Cancer Res* 2000;60(24):6950–6957. [PubMed: 11156395]
19. Palmer GM, Ramanujam N. Monte Carlo-based inverse model for calculating tissue optical properties. Part I: Theory and validation on synthetic phantoms. *Appl Opt* 2006;45(5):1062–1071. [PubMed: 16512550]
20. Palmer GM, Zhu C, Breslin TM, Xu F, Gilchrist KW, Ramanujam N. Monte Carlo-based inverse model for calculating tissue optical properties. Part II: Application to breast cancer diagnosis. *Appl Opt* 2006;45(5):1072–1078. [PubMed: 16512551]
21. Palmer GM, Ramanujam N. Monte-Carlo-based model for the extraction of intrinsic fluorescence from turbid media. *Journal of Biomedical Optics* 2008;13(2):024017–024019. [PubMed: 18465980]
22. Bender JE, Vishwanath K, Moore LK, Brown JQ, Chang V, Palmer GM, Ramanujam N. A Robust Monte Carlo Model for the Extraction of Biological Absorption and Scattering in Vivo. *IEEE Trans BME*. 2008 Accepted for Publication.
23. Zhu C, Palmer GM, Breslin TM, Harter J, Ramanujam N. Diagnosis of breast cancer using fluorescence and diffuse reflectance spectroscopy: a Monte-Carlo-model-based approach. *J Biomed Opt* 2008;13(3):034015. [PubMed: 18601560]
24. Lazebnik M, Zhu C, Palmer GM, Harter J, Sewall S, Ramanujam N, Hagness SC. Electromagnetic spectroscopy of normal breast tissue specimens obtained from reduction surgeries: comparison of optical and microwave properties. *IEEE Trans Biomed Eng* 2008;55(10):2444–2451. [PubMed: 18838370]
25. Skala MC, Palmer GM, Vrotsos KM, Gendron-Fitzpatrick A, Ramanujam N. Comparison of a physical model and principal component analysis for the diagnosis of epithelial neoplasias in vivo using diffuse reflectance spectroscopy. *Opt Express* 2007;15(12):7863–7875. [PubMed: 17912337]
26. Millon SR, Roldan-Perez KM, Riching KM, Palmer GM, Ramanujam N. Effect of optical clearing agents on the in vivo optical properties of squamous epithelial tissue. *Lasers Surg Med* 2006;38(10):920–927. [PubMed: 17163473]
27. Zhu C, Palmer GM, Breslin TM, Harter J, Ramanujam N. Diagnosis of breast cancer using diffuse reflectance spectroscopy: Comparison of a Monte Carlo versus partial least squares analysis based feature extraction technique. *Lasers Surg Med* 2006;38(7):714–724. [PubMed: 16799981]
28. Amelink A, Sterenborg HJB, P. M, Burgers SA. In vivo measurement of the local optical properties of tissue by use of differential path-length spectroscopy. *Opt Lett* 2004;29(10):1087–1089. [PubMed: 15181994]
29. Prahl, S. Optical absorption of hemoglobin. 2008. <http://omlc.ogi.edu/spectra/hemoglobin><http://omlc.ogi.edu/spectra/hemoglobin>
30. Huang, R.; Jacques, S. Skin Optics Summary. 1998. <http://omlc.ogi.edu/news/jan98/skinoptics.html><http://omlc.ogi.edu/news/jan98/skinoptics.html>
31. van Veen RLP, Amelink A, Menke-Pluymers M, van der Pol C, Sterenborg HJCM. Optical biopsy of breast tissue using differential path-length spectroscopy. *Physics in Medicine and Biology* 2005;50(11):2573–2581. [PubMed: 15901955]
32. de Juan A, Tauler R. Chemometrics applied to unravel multicomponent processes and mixtures: Revisiting latest trends in multivariate resolution. *Analytica Chimica Acta* 2003;500(12):195–210.
33. Vaupel P. Hypoxia and Aggressive Tumor Phenotype: Implications for Therapy and Prognosis. *Oncologist* 2008;13(suppl3):21–26. [PubMed: 18458121]
34. Han, J.; Kamber, M. Data mining : concepts and techniques. Elsevier; San Francisco, CA: 2006.
35. Ramanujam, N. Encyclopedia of Analytical Chemistry. Meyers, R., editor. John Wiley and Sons, Ltd.; 2000. p. 20-56.
36. Kong G, Anyarambhatla G, Petros WP, Braun RD, Colvin OM, Needham D, Dewhirst MW. Efficacy of Liposomes and Hyperthermia in a Human Tumor Xenograft Model: Importance of Triggered Drug Release. *Cancer Res* 2000;60(24):6950–6957. [PubMed: 11156395]
37. Ponce AM, Viglianti BL, Yu DH, Yarmolenko PS, Michelich CR, Woo J, Bally MB, Dewhirst MW. Magnetic resonance imaging of temperature-sensitive liposome release: Drug dose painting and antitumor effects. *Journal of the National Cancer Institute* 2007;99(1):53–63. [PubMed: 17202113]

Figure 1.

Diffuse reflectance and fluorescence spectra acquired for a representative animal receiving LTSL-doxorubicin+HT treatment. Plots are shown before and after treatment (1 hour time point). Dashed lines indicate the modeled data, i.e. the Monte Carlo model fits for the diffuse reflectance, and the intrinsic fluorescence spectra. In the case of fluorescence, the data are normalized to the mean value of the pre-treatment spectra in order to display all of the data on the same scale, while preserving differences upon treatment. Diffuse reflectance intensity can be seen to decrease, indicative of an increase in blood volume, while fluorescence at longer wavelength increases, possibly indicative of doxorubicin fluorescence.

Figure 2.

Doxorubicin concentration assessed by fluorescence techniques is strongly correlated with that measured by HPLC ($r=0.88$, $p=7e-21$). A linear least squares trend line is also shown. The primary (heated) side can be seen to have the highest drug accumulation, with most of those with the highest drug concentration belonging to the LTSL-doxorubicin+HT treatment group.

Figure 3.

Box plots showing selected optical properties, namely (a) doxorubicin concentration measured optically, (b) total hemoglobin, and (c) hemoglobin saturation. Within each panel, there are six sub-panels corresponding to the six treatment groups, with each property displayed as a function of time for each group, starting with the baseline, pretreatment value ($t=0$). 1/24 indicates the 1 hour time point immediately post-treatment. Some outliers are excluded from the plots to enable better visualization of the data as a whole.

Figure 4.

Results of the k-means cluster analysis of the hemoglobin saturation and total hemoglobin, measured at baseline for all animals. Two clusters were identified, cluster 1 having relatively low oxygenation and low hemoglobin content, with cluster 2 having the opposite (Fig. 4(a)). It was found that overall there was a significantly worse prognosis for cluster 1 (hypoxic), relative to the control ($p=0.003$). Figure 4(b) shows a survival curve for the two physiologic clusters, indicating that the more hypoxic cluster has a worse prognosis. Figure 4(c) shows box plots of the time to reach 3 times pre-treatment volume for the two clusters, indicating that for most groups, the median time is longer in cluster 2, indicating a better prognosis. Figure 4(d-e) show the highly significant correlations between pre-treatment Hb saturation and time to 3X treatment volume, and the post-treatment total Hb and doxorubicin concentration ($r=0.8$, $p=0.01$, and $r=0.89$, $p=0.001$, respectively, by Spearman rank correlation)

Table 1

Cox proportional hazards ratio for parameters on time to reach 3 times pre-treatment volume.

Predictor	Type of variable or cut point	Hazard ratio (95% confidence interval)	P-value
Hb saturation pre-treatment	>15.1% versus ≤15.1%	0.76(0.34-1.68)	0.49
Hb saturation post-treatment	>23.7% versus ≤23.7%	1.0(0.55-1.82)	1.0
Total Hb pre-treatment	>33.8 μM versus ≤33.8 μM	0.44(0.20-0.98)	0.04
Total Hb post	>42.6 μM versus ≤42.6 μM	0.48(0.26-0.88)	0.017
Doxorubicin Concentration	>1.0 mg/ml versus ≤1.0 μg/ml	0.34(0.19-0.64)	0.0007