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Oxaliplatin-DNA Adducts as Predictive Biomarkers of FOLFOX Response in Colorectal Cancer: A Potential Treatment Optimization Strategy

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Abstract

FOLFOX is one of the most effective treatments for advanced colorectal cancer (CRC). However, cumulative oxaliplatin neurotoxicity often results in halting the therapy. Oxaliplatin functions predominantly via the formation of toxic covalent drug-DNA adducts. We hypothesize that oxaliplatin-DNA adduct levels formed in vivo in peripheral blood mononuclear cells (PBMC) are proportional to tumor shrinkage caused by FOLFOX therapy. We further hypothesize that adducts induced by sub-therapeutic “diagnostic microdoses” are proportional to those induced by therapeutic doses and are also predictive of response to FOLFOX therapy. These hypotheses were tested in CRC cell lines and a pilot clinical study. Four CRC cell lines were cultured with therapeutically relevant (100 μM) or diagnostic microdose (1 μM) concentrations of [^{14}C]oxaliplatin. The C-14 label enabled quantification of oxaliplatin-DNA adduct level with accelerator mass spectrometry (AMS). Oxaliplatin-DNA adduct formation was correlated with oxaliplatin cytotoxicity for each cell line as measured by the MTT viability assay. Six CRC patients received by IV a diagnostic microdose containing [^{14}C]oxaliplatin prior to treatment, as well as a second [^{14}C]oxaliplatin dose during FOLFOX chemotherapy, termed a “therapeutic dose.” Oxaliplatin-DNA adduct levels from PBMC correlated significantly to mean tumor volume change of evaluable target lesions (5 of the 6 patients had measurable disease). Oxaliplatin-DNA

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adduct levels were linearly proportional between microdose and therapeutically relevant concentrations in cell culture experiments and patient samples, as was plasma pharmacokinetics, indicating potential utility of diagnostic microdosing.

Keywords

diagnostic microdosing; chemotherapy resistance; colorectal cancer; accelerator mass spectrometry; FOLFOX; oxaliplatin

Introduction

Colorectal cancer (CRC) is the third most frequent cancer and is a leading cause of cancer related death worldwide (1). Modern treatments for CRC may include surgery, radiation, chemotherapy and targeted therapy. Whereas the complete surgical removal of the tumor is considered the mainstay treatment for early tumors (Stages I+II), systemic administration of cytotoxic chemotherapy predominates for late stage disease. Several major advances have been made over the past fifteen years resulting in improved overall survival. In large part, these advances are based on the approval of two cytotoxic therapies, oxaliplatin and irinotecan, which can be used in combination regimens with an older standard chemotherapy drug 5-fluorouracil. However, it remains an important need to maximize efficacy and minimize toxicities of those cytotoxic regimens to realize further extension of progression free survival (PFS) and overall survival (OS) in patients with metastatic CRC. Since its approval in 2004, FOLFOX, consisting of leucovorin (folic acid), 5-fluorouracil and oxaliplatin, has been widely adopted as a standard treatment in CRC in both adjuvant and metastatic settings. Sensory neuropathy is the major dose limiting toxicity of oxaliplatin and occurs with consecutive and cumulative dose received. Due to function limiting sensory neuropathy, patients often require dose adjustment or discontinutation of oxaliplatin in the absence of disease progression. Various strategies of intermittent FOLFOX use and FOLFOX rechallenge have been employed to maximize oxaliplatin exposure in metastatic CRC (1–3). As a result, many CRC patients will be exposed and re-exposed to oxaliplatin throughout their treatment course until resistance and toxicities develop. A difference in oxaliplatin absorption, metabolism and DNA repair processes in individual patients potentially alters the accumulation of oxaliplatin in tumor and normal tissue DNA which may affect efficacy and toxicities of oxaliplatin, respectively. A biomarker to predict the optimal dose of oxaliplatin in an individual patient which maximizes efficacy while potentially minimizing toxicity is a clear unmet need. The cytotoxicity of oxaliplatin is predominantly a consequence of the formation of covalent drug-DNA adducts (Figure 1)(4–6). Multiple biological pathways such as drug transport, metabolism, DNA damage repair, and cell cycle modulation affect the activity, or efficacy of oxaliplatin (2, 7). Molecular pathway analysis has resulted in a better understanding of these highly complex resistance mechanisms, but these results have yet to be translated into clinical utility.

This complexity motivated us to investigate the possible utilization of microdose-induced drug-DNA adducts as functional biomarkers for oxaliplatin response in CRC. The sub-therapeutic “diagnostic microdosing” approach has been reported to potentially provide

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predictive information regarding chemosensitivity to platinum-based chemotherapy (5, 8, 9). This approach uses the tracing of non-toxic diagnostic microdoses of ^{14}C-labeled oxaliplatin via accelerator mass spectrometry (AMS), which has attomole (10^{-18} mole) sensitivity for ^{14}C; easily sensitive enough for clinical applications. AMS provides an isotope ratio for each DNA sample that allows calculation of the drug concentration in tissues, blood, protein or nucleic acids at concentrations that are difficult or impossible to measure with other techniques. AMS therefore enables human studies with radiolabeled drug while circumventing the need for toxic drug or high radiation exposures (10, 11). We hypothesize that DNA damage caused by a single sub-toxic microdose of oxaliplatin can predict patient outcomes such as tumor shrinkage and survival. We report herein investigation of oxaliplatin microdosing with four CRC cell lines and six CRC patients. The goal of the project was to define the appropriate chemical and radiochemical dose for the microdose composition, to establish protocols for the procedure and to gather preliminary clinical data in support for a larger clinical trial.

Materials and Methods

Chemicals

Unlabeled oxaliplatin (5 mg/mL) was purchased from Sanofi-Aventis (Bridgewater, NJ). ^{14}C-labeled oxaliplatin (specific activity 54 mCi/mmol with the ^{14}C-label in the diaminocyclohexane (DACH) group) was obtained from Moravek Biochemicals (Brea, CA). [^{14}C]oxaliplatin for injection was prepared under good manufacturing practice (GMP) at the GMP facility at UC Davis. The [^{14}C]oxaliplatin drug substance was dissolved with sterile water for injection (APP Pharmaceuticals, Schaumburg, IL). The solution was filter sterilized with a 0.2 μm PES syringe filter and the resulting drug product was stored at −20°C. Specific activity was determined by liquid scintillation counting (LSC). Mixtures of ^{14}C-labeled and unlabeled drug were used to minimize the usage of radiocarbon and achieve the different specific activities required for microdoses and therapeutic doses. Drug solutions for the indicated experiments were prepared immediately before use.

Cell lines and cytotoxicity assay

Four human CRC cell lines (CLL-228, CLL-229, CRL-2134 and HBT-38) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured with the recommended medium without additional verification of identity. The IC_{50} values were determined in triplicate via standard MTT assay as described elsewhere (12). The MTT assay and diagnostic microdoses were performed on cell lines with as close as possible to the same number of passages. Therefore, cell cultures were continuously exposed to oxaliplatin for 72 hours before assay development.

Drug treatment and AMS analysis

Using a protocol derived from previously reported [^{14}C]carboplatin microdosing studies (9, 13, 14), cells were cultured to >90% confluence, dosed with indicated doses of [^{14}C]oxaliplatin, further cultured for up to 24h, and subjected to DNA isolation and AMS analysis as previously described (14). Briefly, cells were seeded at 1 x 10^6 cells per 60 mm dish and allowed to attach overnight at 37°C and 5% CO_2 in a humidified atmosphere. At

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hour 0, cells were dosed with 1 μM (microdose) or 100 μM oxaliplatin (therapeutic dose), each supplemented with [^{14}C]oxaliplatin at 500 dpm/mL. Cultures were incubated for 4h then washed twice with phosphate-buffered saline (PBS) and maintained thereafter with drug-free culture media for indicated periods of time to mimic the human oxaliplatin plasma half-life (0.5-16 hours) and capture possible DNA repair kinetics (15). Cells were harvested at 0, 2, 4, 8 and 24 hours after initiation of dosing. DNA was extracted from collected cells with the Wizard Genomic DNA Purification Kit according to the manufacturer's instruction (Promega, Madison, WI, USA). DNA quantity and quality was determined with a NanoDrop 1000 spectrophotometer, the purity was ensured by obtaining a 260/280 nm OD ratio of approximately 1.9. The protocol included washing of the DNA pellet with aqueous alcohol solutions in order to eliminate contamination by proteins and small molecule metabolites. Assessing the 260/280 nm OD ratio minimized the possibility of contamination of the samples with RNA. All DNA samples were submitted to Lawrence Livermore National Laboratory (LLNL) for AMS analysis of radiocarbon content using an established protocol (16). Ten micrograms of DNA per sample was converted to graphite and measured by AMS for ^{14}C quantification as previously described. Triplicate sets of AMS experiments were performed for each cell line and time point. The data was plotted as oxaliplatin-DNA adducts per 10^8 nucleotides (nt) over time.

A pilot feasibility diagnostics clinical trial

The study titled "Pilot Study of a Carbon 14 Oxaliplatin Microdosing Assay to Predict Exposure and Sensitivity to Oxaliplatin-Based Chemotherapy in Advanced Colorectal Cancer" (ClinicalTrials.gov identifier NCT02569723) was a feasibility study of the diagnostic microdosing approach and was approved by the UC Davis Institutional Review Board and conducted under an IND from FDA. The study was conducted in accordance with the Declaration of Helsinki and performed after UC Davis Institutional Review Board approval and after obtaining written informed consent from the subjects. The study included patients with locally advanced or metastatic colorectal adenocarcinoma. The study design was derived from a previous carboplatin-based microdosing pilot clinical study that accrued bladder and lung cancer patients, but with an emphasis on correlation of microdose-induced drug-DNA adduct levels to mean tumor volume change rather than response as defined by RECIST criteria (9, 13). The therapeutic dose of oxaliplatin was administered at 85 mg/m^2. Diagnostic microdoses of oxaliplatin were administered to patients at 1% of the therapeutic dose. [^{14}C]oxaliplatin was given at 2 x 10^6 dpm/kg. The ^{14}C containing microdose and therapeutic dose were administered by a 2 hour intravenous infusion (IV). Toxicity of the two administered dose levels (microdose and therapeutic dose) was assessed using Common Terminology Criteria for Adverse Events (CTCAE). Patient response to FOLFOX chemotherapy was evaluated using the RECIST and correlated to oxaliplatin-DNA adduct levels. Unlabeled oxaliplatin and [^{14}C]oxaliplatin were administered unmixed but simultaneously and peripheral blood specimens from a separate access point were drawn into BD Vacutainer CPT™ tubes with sodium citrate (Becton Dickinson). Peripheral blood mononuclear cells (PBMC) were isolated within 2 hours of collection by centrifugation according to manufacturer's instruction. A proportion of ultra-centrifuged (10 kDa MWCO) and total plasma were used for liquid scintillation counting (LSC).

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LC-MS

Human plasma samples were made for establishing a calibration curve. For each sample, 50 μL of oxaliplatin (Selleckchem.com, Houston, TX) solution was prepared at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 μg/mL in H_2O (Fisher Scientific, Hampton, NH), and were each spiked with 450 μL of pooled blank human plasma in sodium citrate buffer (Bioreclamation IVT, Westbury, NY). Quality control (QC) solutions were made by mixing of 50 μL of oxaliplatin stock solution at 0.025, 0.25, and 25 μg/mL in H_2O with 450 μL of blank human plasma. The resulting human plasma calibration standards, QC samples and the patient plasma samples were filtered with 10 kDa MWCO ultrafiltration filters (EMD Millipore, Billerica, MA) prior to LC/MS analysis. Twenty microliters of each resulting filtrate was spiked with 80 μL of 1 μg/mL carboplatin (Hospira Inc, Lake Forest, IL) in H_2O, which served as an internal standard. Five microliter of the resulting mixture was injected into a Waters (Milford, MA) Acquity UPLC with a BEH C18 1.7 μm, 2.1 mm × 50 mm column. The following mobile phases were used: A) H_2O with 0.1% formic acid (Fisher Scientific, Hampton, NH), and B) methanol (Fisher Scientific, Hampton, NH) with 0.1% formic acid. Mobile phase A was also used for purging and needle washing between each injection. The flow rate was 0.4 mL/min and the column temperature was 50°C with an autosampler temperature of 10°C. The output of the UPLC was fed to a Waters Xevo TQ-S triple quadrupole MS/MS system, which was used to ionize target molecules and monitor the ion m/z fragmentation transitions from 398.3 → 96.1 for oxaliplatin quantification, and 371.9 → 294.2 for carboplatin at multiple reaction monitoring (MRM) mode. The retention times were 1.02 and 0.70 minutes for oxaliplatin and carboplatin, respectively. The detection range for oxaliplatin was from 0.5 to 5000 ng/mL. The calibration curve was modeled with a weighted (1/x2) least-squares linear regression algorithm. The lower limit of quantification (LLOQ) was 0.5 ng/mL. The extraction yield for oxaliplatin was 73.67 ± 8.12% and the matrix effect enhanced the oxaliplatin MS signal by 16.71%. Both inter and intra batch accuracy were lower than 10% (% deviation) and both intra and inter batch precision were also lower than 10% (%CV).

Statistical analysis

Statistical analysis was performed using GraphPad Prism™ software (GraphPad Software Inc., CA, USA) using a two tailed *t*-test or a 1-way ANOVA with Bonferroni's *post hoc* test, as appropriate. A *p*-value below 0.05 was considered statistically significant. All experiments were carried out at least in triplicate in order to enable statistically significant comparisons of the results. All results are expressed as the mean ± SD unless otherwise noted. A simple correlation of the adduct levels and tumor volume change is reported.

Results

Oxaliplatin-DNA adduct levels induced by diagnostic microdose and therapeutic concentrations in CRC cell cultures are dose proportional

We tested the hypothesis that the levels of microdose induced oxaliplatin-DNA adducts in CRC cell cultures are proportional to those induced by therapeutic doses. Four CRC cell lines were exposed to therapeutically relevant (100 μM) or diagnostic microdose (1 μM) concentrations of [^{14}C]oxaliplatin for 4 hours, followed by incubation in drug-free medium.

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Cells were harvested at various time points over a period of 24 hours (Figures 1+2) and drug-DNA adduct levels were determined through AMS analysis of purified genomic DNA. The dosing protocol was designed to crudely mimic *in vivo* cell exposure to oxaliplatin (exponential decrease of drug concentration in blood over approximately a day). As expected, there was a time-dependent increase in oxaliplatin-DNA adduct levels during the first 4 hours (Figure 2) of drug exposure to either concentration followed by a gradual decrease over the subsequent 20 hours of cell incubation in drug-free medium. The peak-adduct levels varied considerably between the different cell lines even though all cell lines showed the same overall trend of adduct concentration peak and decline over time. The mean oxaliplatin-DNA adduct levels of across all cell lines from microdose administration (1-10 adducts/10^8 nt) were approximately 100-fold lower than the levels obtained from therapeutic dosing (100-1,000 adducts/10^8 nt, Figure 2C). Linear regression analysis showed that the adduct levels induced by the two oxaliplatin dose-concentrations were highly linear and correlated significantly ($R^2 = 0.95$, $p < 0.0001$, Figure 2D), which supports the concept that microdosing can be used to predict the DNA adduct levels induced by therapeutic oxaliplatin without negatively impacting cell viability.

Genomic drug-DNA adduct level correlate with CRC cell sensitivity to oxaliplatin

We next evaluated if the sensitivity of CRC cell cultures to oxaliplatin can be determined by analysis of drug-DNA adduct formation. Cell sensitivity towards oxaliplatin was determined via the MTT viability assay after a 72h oxaliplatin exposure. The four tested cell lines were allocated into either sensitive or the resistant groups (two cell lines in each group based on relative oxaliplatin sensitivity). At each time point the two most resistant cell lines (CLL-228 and CLL-229) had lower oxaliplatin-DNA adduct levels than the two most sensitive cell lines (CRL-2134 and HTB-38) (Figure 3A+B and Table 1). There was a significant difference between the two groups after 4h exposure to the microdose ($p < 0.01$) or therapeutic dose ($p < 0.05$). The mean area under the adduct curve ($AUC_{adducts}$) of the two resistant cell lines was lower than the $AUC_{adducts}$ of the sensitive cell lines but did not reach significance (Supplemental Figure 1).

A pilot diagnostic feasibility trial in human CRC patients

Our main objective was to test the feasibility of using [^{14}C]oxaliplatin diagnostic microdoses to enable prediction of therapeutic oxaliplatin exposure, pharmacodynamics (drug-DNA adduct levels) and corresponding tumor volume changes. The study utilized the assessment of oxaliplatin-DNA adduct levels as biomarkers in genomic DNA from PBMC as surrogates for tumor biopsy samples and also examined the intra-patient variability of micro- and therapeutic dose pharmacokinetics. We tested the hypotheses that the intravenous infusion of a diagnostic [^{14}C]oxaliplatin microdose will predict the pharmacokinetic exposure to treatment dose oxaliplatin and that accumulation of the microdose-induced oxaliplatin-DNA damage levels in PBMC will correlate with patient response to subsequent full-dose oxaliplatin-based chemotherapy.

The clinical feasibility trial was initiated at UC Davis Comprehensive Cancer Center (ClinicalTrials.gov identifier NCT02569723) and a total of 6 patients with locally advanced or metastatic colorectal adenocarcinoma were accrued. The patients that completed the

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diagnostic microdosing and blood sampling, also received an additional [^{14}C]oxaliplatin dose with their subsequent full dose FOLFOX, which is referred to herein as a therapeutic dose of [^{14}C]oxaliplatin.

Similar to what was observed clinically in bladder and non-small cell lung cancer (9, 13), the microdose composition of [^{14}C]oxaliplatin of 2x10^{6} dpm/kg and a total oxaliplatin dose of 1% of the therapeutic dose, the ^{14}C-signal in DNA isolated from PBMC was approximately 10-100 times the background, which allowed accurate adduct measurement by AMS. As expected, no microdose-associated adverse events were observed in any of the patients. The administered diagnostic [^{14}C]oxaliplatin microdose appears to be safe in this patient population. In comparison to the annual effective radiation dose equivalent from natural internal sources of 1.6 mSv per person, (17) and a radiation exposure for an abdominal CT scan of 10 mSv, (18) the average administration of the diagnostic microdose was not greater than 0.01 mSv.

Linear correlation of oxaliplatin pharmacokinetics between microdose and therapeutic dose

Immediately following the blood draws, whole plasma was isolated for PK analysis via liquid scintillation counting (LSC) and liquid chromatography mass spectrometry (LC-MS). The total and free oxaliplatin plasma concentration after diagnostic microdoses (Figure 4A–C) and therapeutic doses (Figure 4D–F) showed the expected increase over the 2h IV infusion and an approximately biphasic elimination kinetic in the following 22 to 46 hours. At 24 hours after dosing, only 50-75% of total ^{14}C label was cleared from whole plasma (Figure 4A+D). Analysis of free [^{14}C]oxaliplatin after removal of plasma proteins by 10 kDa MWCO filtration showed that at 24h less than 10% of the [^{14}C]oxaliplatin was detectable (Figure 4B+E). The expected discrepancy between the ^{14}C concentrations in total and ultra-centrifuged plasma has been previously reported and is closely linked to oxaliplatin's high binding probability to plasma proteins and erythrocytes (15). To further validate the approach of determining [^{14}C]oxaliplatin PK with LSC, we used LC-MS to determine the oxaliplatin concentration in 10 kDa MWCO filtrated patient plasma specimen (Figure 4C+G). Analysis of free oxaliplatin plasma concentrations after micro- or therapeutic doses via LSC (Figure 4D) and LC-MS (Figure 4H) shows a highly significant linear correlation ($R^2 = 0.94$, $p < 0.0001$ and $R^2 = 0.68$, $p < 0.0001$, respectively). As expected, the LC-MS method was less sensitive compared to LSC and AMS, since several time points were below the lower limit of detection due to the very low (sub-therapeutically) oxaliplatin concentration in the diagnostic microdose.

Correlation of microdose-induced oxaliplatin-DNA monoadducts in PBMC with tumor response to subsequent full dose FOLFOX chemotherapy

A key aspect of this study is that the level of oxaliplatin-DNA adducts in PBMC was used as a surrogate biomarker for analysis of tumor DNA. This approach was chosen due to limited access to biopsy samples from metastatic CRC patients. This choice is also justified by several studies by us and others that showed that platinum-based drug-DNA adducts in PBMC correlate with tumor response to chemotherapy (5, 6, 8, 9, 19–23). Even though most of the free [^{14}C]oxaliplatin was cleared from blood within 24h, adduct levels were readily

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measurable for up to 48 hours. The oxaliplatin-DNA adduct levels following the diagnostic microdose or therapeutic dose ranged from 0.1-6 and 13-1047 adducts per 10^8 nucleotides, respectively (Figure 5A+B and table 2). Mean oxaliplatin-DNA adduct levels across all microdosed patients were approximately 100-fold lower than those observed after therapeutic dosing (Figure 5C). Linear regression analysis showed that the adduct levels induced by the two oxaliplatin dose-concentrations were highly linear and correlated significantly ($R^2 = 0.67$, $p < 0.0001$, Figure 5D), suggesting that the nontoxic diagnostic microdosing approach can be used to predict the DNA adduct levels induced by therapeutic oxaliplatin without negatively impacting patient health or PBMC viability.

The clinical response of the CRC patients was determined by measuring the percent change in the sum of the target lesions (measurable disease) and is shown in Table 2. Measurable disease data were collected from 5 of the 6 patients accrued. Correlation analysis of the mean change in target lesions after 3-4 cycles of FOLFOX with induced microdose (Figure 5E) or therapeutic dose (Figure 5F) adduct levels at 4h shows a linear relationship ($R^2 = 0.082$, $p = 0.033$, $R^2 = 0.62$, $p = 0.099$, respectively). Patients responded to FOLFOX chemotherapy to varying degrees with the patient achieving the highest 4h adduct-level yielding almost complete remission of the measured target lesions after 12 cycles of therapy. In contrast, the patient with the lowest 4h adduct level had initially disease progression after 3 cycles as evidenced by a 9% increase in mean tumor volume but showed a modest tumor volume reduction of 6% after 8 cycles. In addition to yielding the best correlations, 4 h time point post microdose is reasonable for clinical implementation of such a diagnostic test since patients would not have to make repeat hospital visits in between dosing and blood sampling. This early trend associating oxaliplatin-DNA adduct level with CRC patient response is encouraging. However, more patients need to be accrued and the protocol needs to be optimized in order to maximize accrual and more closely match how such a test would be utilized in the clinic.

Discussion

The results presented herein indicate that (1) oxaliplatin-DNA adduct levels in colorectal cancer cell lines correlate to cellular sensitivity to the drug, (2) that microdose-induced oxaliplatin-DNA adduct levels in PBMC are predictive of those formed by therapeutic doses in vivo, and (3) that such data are predictive of FOLFOX response, at least for some time points. Collectively, these data point to potential clinical utility for diagnostic microdosing.

CRC patients often respond well to FOLFOX only to discontinue treatment due to cumulative and irreversible oxaliplatin-induced neuropathy. Diagnostic microdosing has the potential to enabled adjustment of the oxaliplatin dose or the frequency of each cycle to target a adduct levels at specific time points or the area under the adduct curve that would enable greater precision FOLFOX. For example, patients that form relatively high oxaliplatin-DNA adducts could be given smaller doses or less frequent cycles of FOLFOX in order to avoid early onset neuropathy while maintaining efficacy. For those patients with relatively low oxaliplatin-DNA adducts, dose dense therapy or alternative chemotherapeutic regimens could be considered to enhance efficacy.

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Tumor-related oxaliplatin resistance is based on a multitude of molecular mechanisms, including alterations in drug transport, detoxification, DNA damage response and repair, cell death (apoptotic and non-apoptotic), and epigenetic mechanisms. Diagnostic microdosing is an attractive concept due to the simplicity of measuring a single phenotypic marker that is influenced by a large array resistance factors, and without the need for a tumor biopsy. Furthermore, measurement of drug-DNA adduct levels may be useful as a tool for better understanding DNA repair pathways of clinical relevance.

We determined the feasibility of a diagnostic microdosing approach for the study of colorectal cancer cellular sensitivity to oxaliplatin. Our data support the concept that microdose-induced oxaliplatin-DNA adduct levels are predictive of cellular sensitivity to oxaliplatin and tumor volume control in CRC patients. This approach is an extension our previous work that focused on bladder and lung cancer, predominantly for cisplatin- and carboplatin-based therapy response prediction, but with extension of the methodology for dosing, sample collection and data analysis for assessment of FOLFOX optimization (9, 13, 14, 24). There was a significant linear correlation between oxaliplatin-DNA adduct levels induced by microdose and therapeutic dose in four CRC cell lines. In addition, cell line sensitivity to oxaliplatin significantly correlated to oxaliplatin-DNA adduct levels after 4 hours of drug exposure with both drug concentrations.

Our preclinical cell line data formed the foundation of the feasibility microdosing trial aimed at determining whether oxaliplatin-DNA adduct levels in PBMC correlate with the response to FOLFOX chemotherapy in CRC patients. Our pilot study showed that a diagnostics feasibility study can be performed in patients without any detectable toxicity associated with the microdose. This pilot study was not designed to demonstrate statistical significance of drug-DNA adduct frequency as a biomarker, but still yielded some encouraging results (Figure 5).

First, we developed protocols for conducting a clinical trial with a ^{14}C-labeled drug and processing samples for AMS analysis. Based on this effort, we decided to perform liquid biopsies (blood sampling) for up to 48 hours after dosing. At 24 hours after dosing, there were measurable yet large inter-patient variations in drug-DNA adduct levels, while the [^{14}C]oxaliplatin concentration in plasma was negligible by LC-MS.

Second, we defined a clinically useful of oxaliplatin microdose formulation consisting of 1% of the therapeutic dose of oxaliplatin and $2x10^6$ dpm/kg of patient's body weight of labeled drug. We were able to correlate oxaliplatin-DNA adduct levels with clinical response in five out of six patients. One patient with the highest adduct levels after 4h had an almost complete remission response, whereas the patient with the lowest adduct level did respond to FOLFOX, but to a much lesser degree. This early trend associating oxaliplatin-DNA adduct level with CRC patient response is encouraging. However, more patients need to be accrued to determine if adduct levels are dependent upon tissue type and are predictive of clinical response.

Beyond the selection of oxaliplatin-based versus irinotecan-based initial therapy for palliative treatment of advanced CRC, there are multiple clinical scenarios where the

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application of diagnostic microdosing with oxaliplatin has the potential improve therapeutic decision making. For example, an understanding of the potential response to oxaliplatin-based therapy could improve the selection of treatment in patients with colorectal cancer liver metastases being considered for treated with intensive regimens such as FOLFOXIRI plus bevacizumab (25). Additionally, variation in oxaliplatin-DNA adduct formation could potentially be used to optimize the selection of an oxaliplatin-based regimen as compared to gemcitabine and albumin bound paclitaxel amongst patients with pancreatic cancer. Future investigation is required to translate our promising feasibility results to clinical utility in these settings. AMS analysis via conversion to graphite and quantification of the ratio of ^{14}C to total carbon on specialized instrumentation is currently cost prohibitive and low throughput compared to the potential clinical need for many thousands of samples per year. However, the throughput and technology are improving with the recent advent of gas ionization sources and even laser-based systems that may render the diagnostic microdosing approach accessible to many laboratories and even hospitals settings.

In conclusion, we developed a highly sensitive AMS-based assay that can possibly identify cellular sensitivity to platinum-based drugs prior to toxic treatment. Based on this pilot study, a diagnostics feasibility clinical trial is currently in planning with enough power for statistical analysis to determine if oxaliplatin-DNA monoadduct levels correlate with chemotherapy sensitivity, which would precede a subsequent study aimed at adjusting patient treatment planning based on the resulting data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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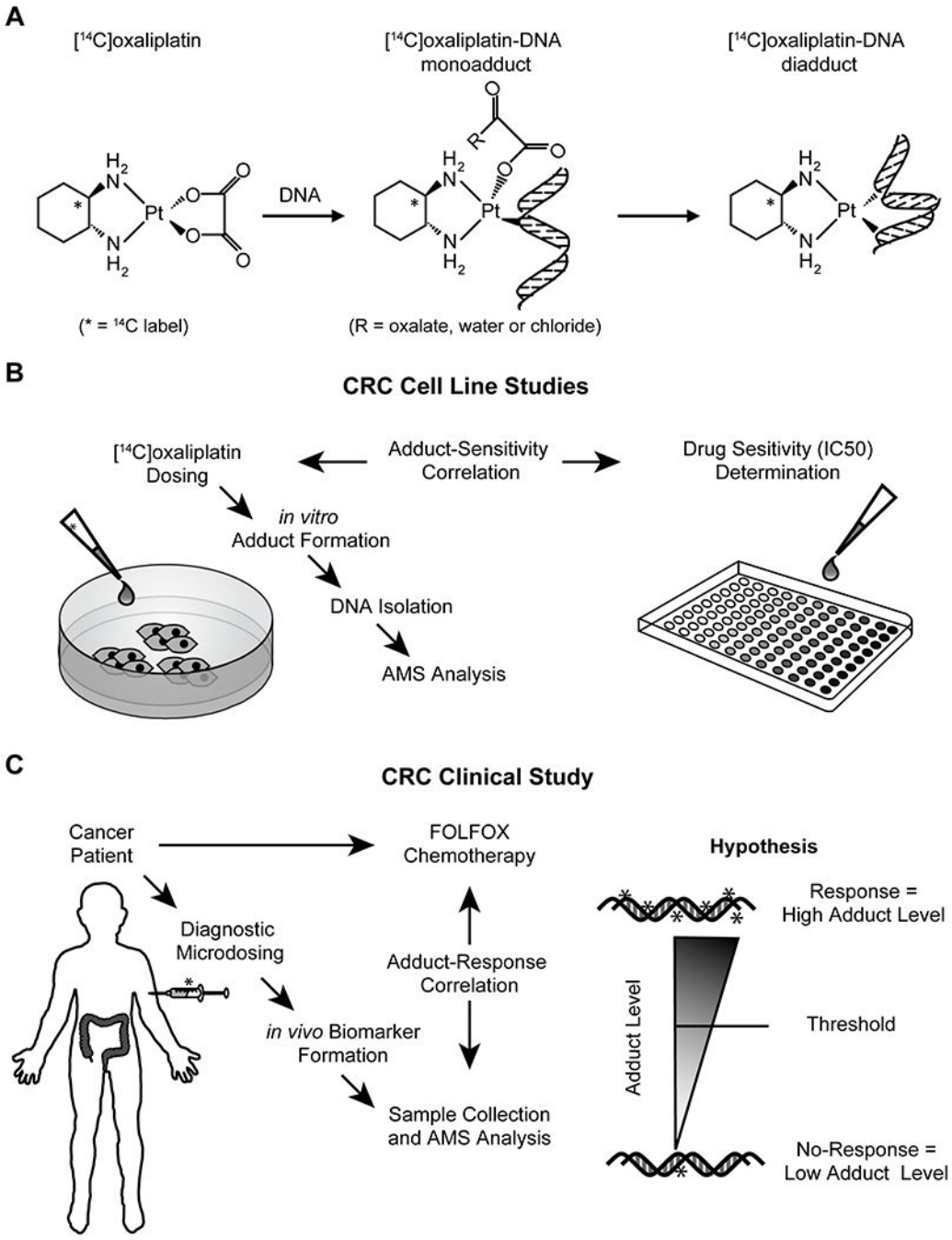

Figure 1. Schematically overview of oxaliplatin-DNA adduct formation and methodology used for *in vitro* or *in vivo* experiments.

A) Simplified scheme for the formation of oxaliplatin-DNA adducts. The ^{14}C label (*) in oxaliplatin is located in the DACH carrier group, therefore both forms of adducts (mono-and diadducts) can be detected by AMS. When adducts are repaired, the ^{14}C label is removed from the DNA and no longer be detected. **B+C)** Illustration of experimental design of *in vitro* or *in vivo* dosing and sensitivity correlation. **B)** CRC cell cultures were exposed to [^{14}C]oxaliplatin and drug-DNA adduct level were assessed via AMS. Drug sensitivity was

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determined via MTT and IC_{50} values were correlated to adduct level. **C)** CRC patients were administered a [^{14}C]oxaliplatin microdose prior to blood sampling and FOLFOX chemotherapy. DNA was then purified from isolated PBMC and assayed for drug-DNA adduct formation via AMS. The drug-DNA adduct levels were correlated to tumor reduction.

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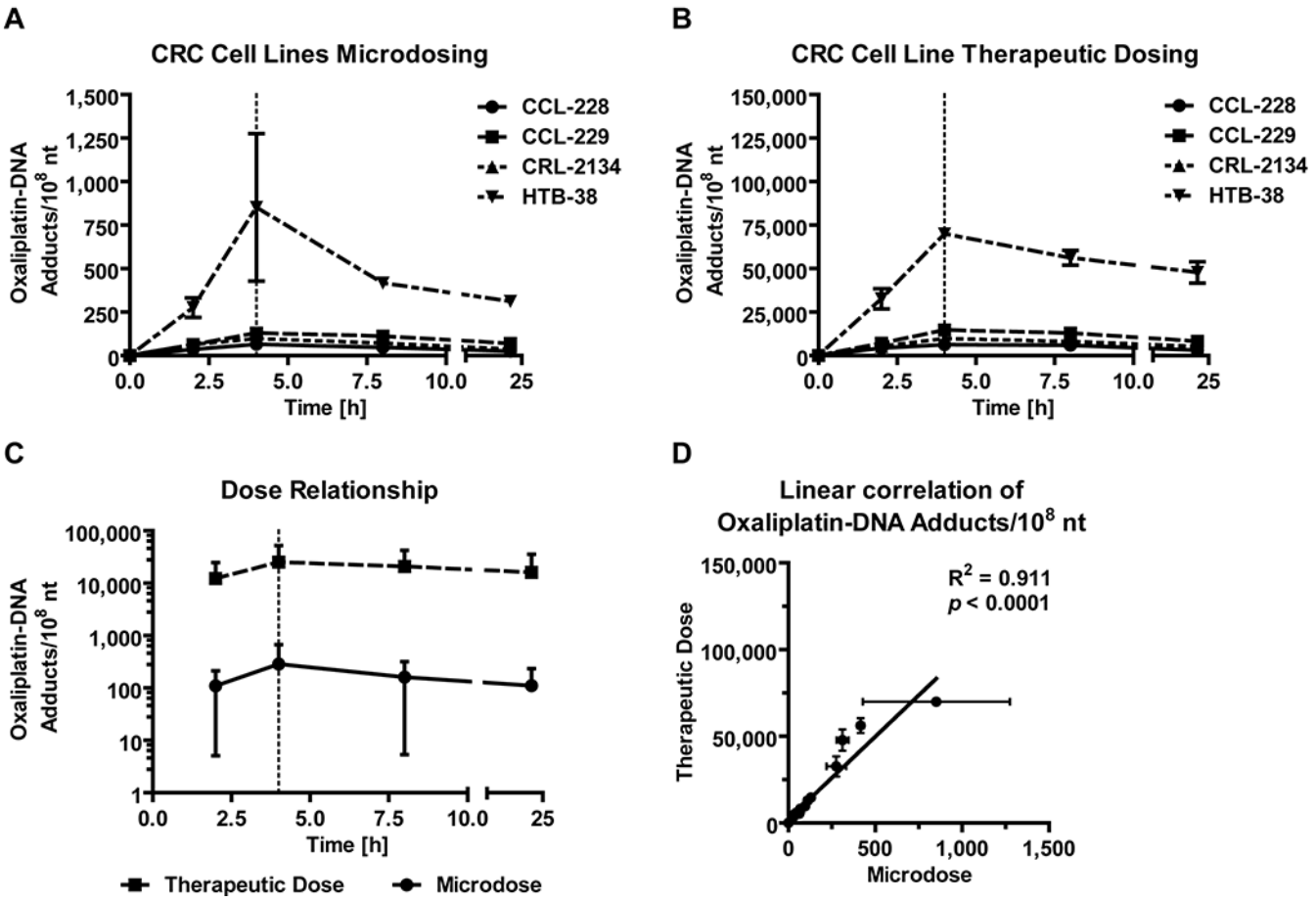

Figure 2. Oxaliplatin-DNA adducts induced by microdose and therapeutic concentrations in CRC cell cultures are dose proportional.

Indicated four CRC cells lines were dosed for 4h followed 20h incubation in drug-free medium. **A**) Microdoses (1 μM) or **B)** therapeutic dose (100 μM) induced oxaliplatin-DNA adduct level over time. **C)** Dose proportionality of microdose and therapeutic dose induced oxaliplatin-DNA adduct level (log scale). **D)** Linear correlation of oxaliplatin-DNA adduct level induced by microdosing and therapeutic oxaliplatin, showing that the adduct levels induced were highly linear ($R^2 = 0.91$, $p < 0.0001$). Mean and standard error are shown.

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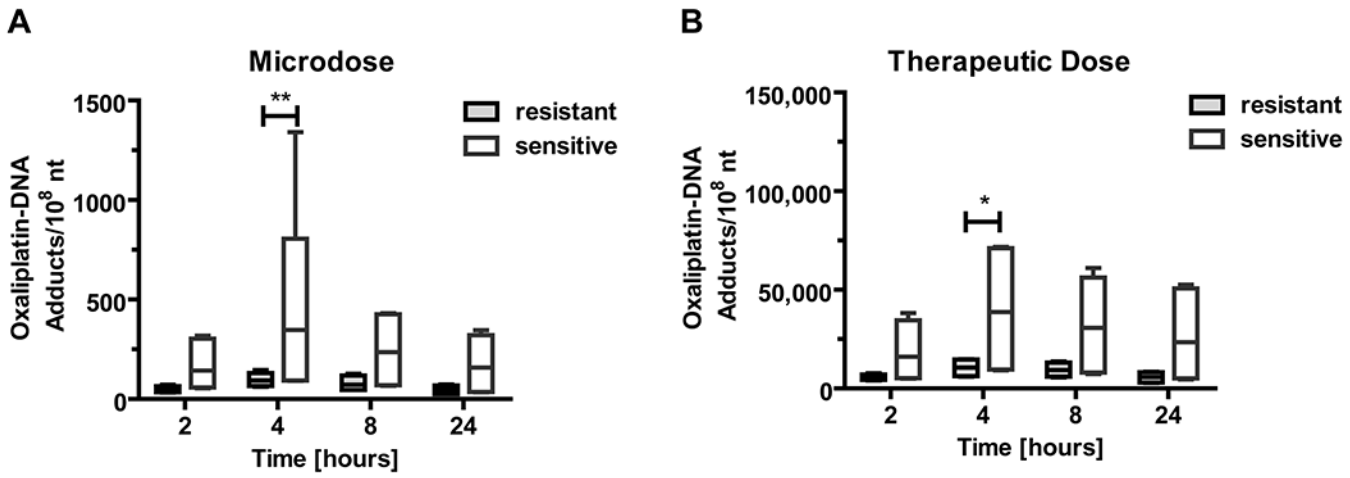

Figure 3. Correlation between CRC cell line oxaliplatin sensitivity and oxaliplatin-DNA adduct levels.

Box plot (whiskers min to max) comparing adduct levels in four CRC cell lines over 24h. Compared to the two most resistant cell lines CLL-228 and CLL-229 (gray box), the two most sensitive cell lines CRL-2134 and HTB-38 (white box) had significant (** = $p < 0.01$, * = $p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test) higher adduct level after 4h of **A** microdose or **B** therapeutic dose oxaliplatin exposure.

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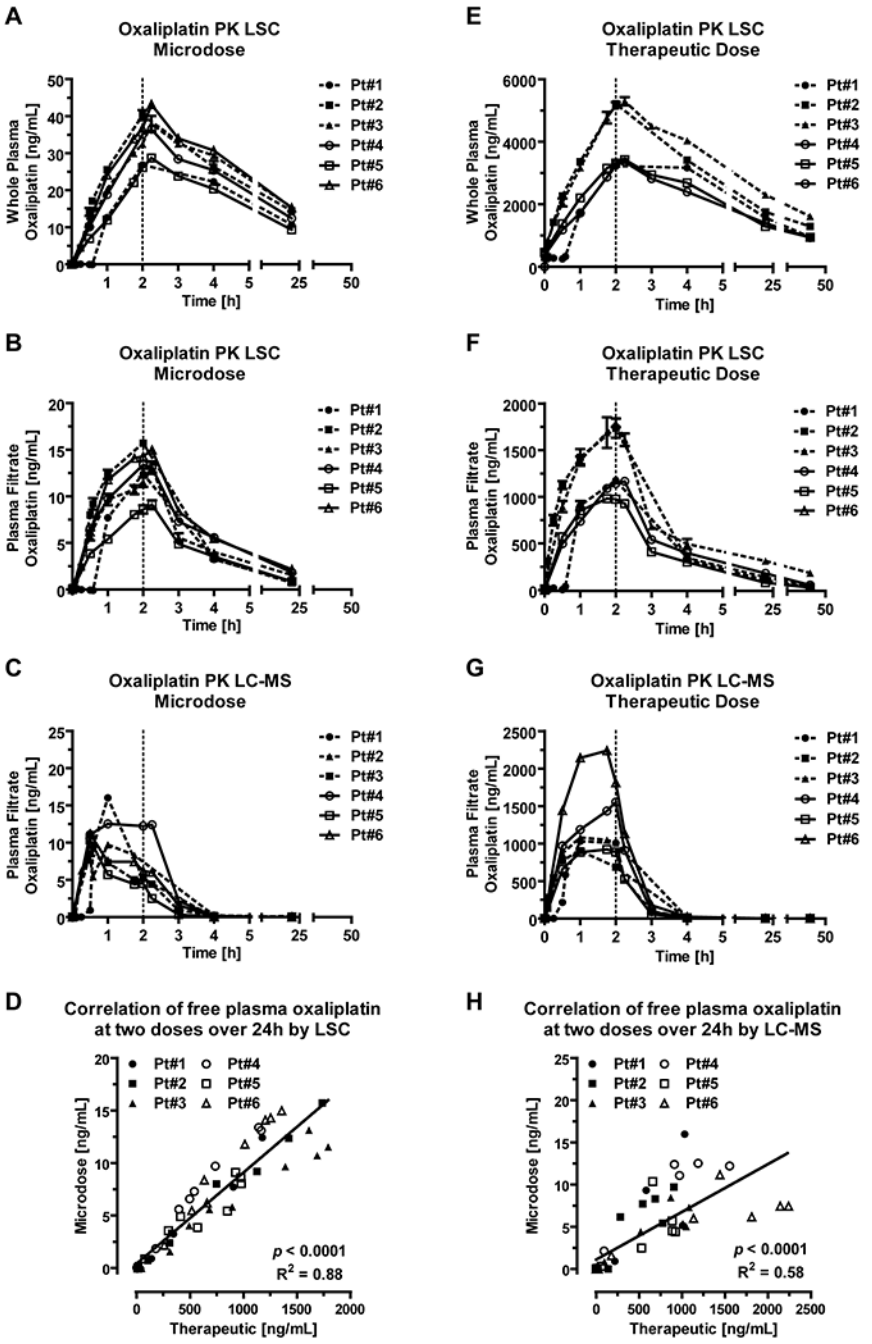

Figure 4. Oxaliplatin plasma concentration after microdose and therapeutic dose infusion in CRC patients are dose proportional.

Indicated three CRC patients (patient #1: Pt1 ●, #2: Pt2 ■, and #3: Pt3 ▲) were infused for 2 h with **A-C)** a microdose or **E-G)** therapeutic dose of oxaliplatin. Whole plasma oxaliplatin concentrations were analyzed over a period of **A)** 24h or **E)** 48h via LSC. Free oxaliplatin concentrations from 10 kDa MWCO centrifuged plasma was determined via **B** +**E**) LSC or **C**+**F)** LC-MS. Mean values and SD are shown. Linear correlation of free

plasma oxaliplatin after microdose and therapeutic dose determined via **D)** LSC or **H)** LC-MS.

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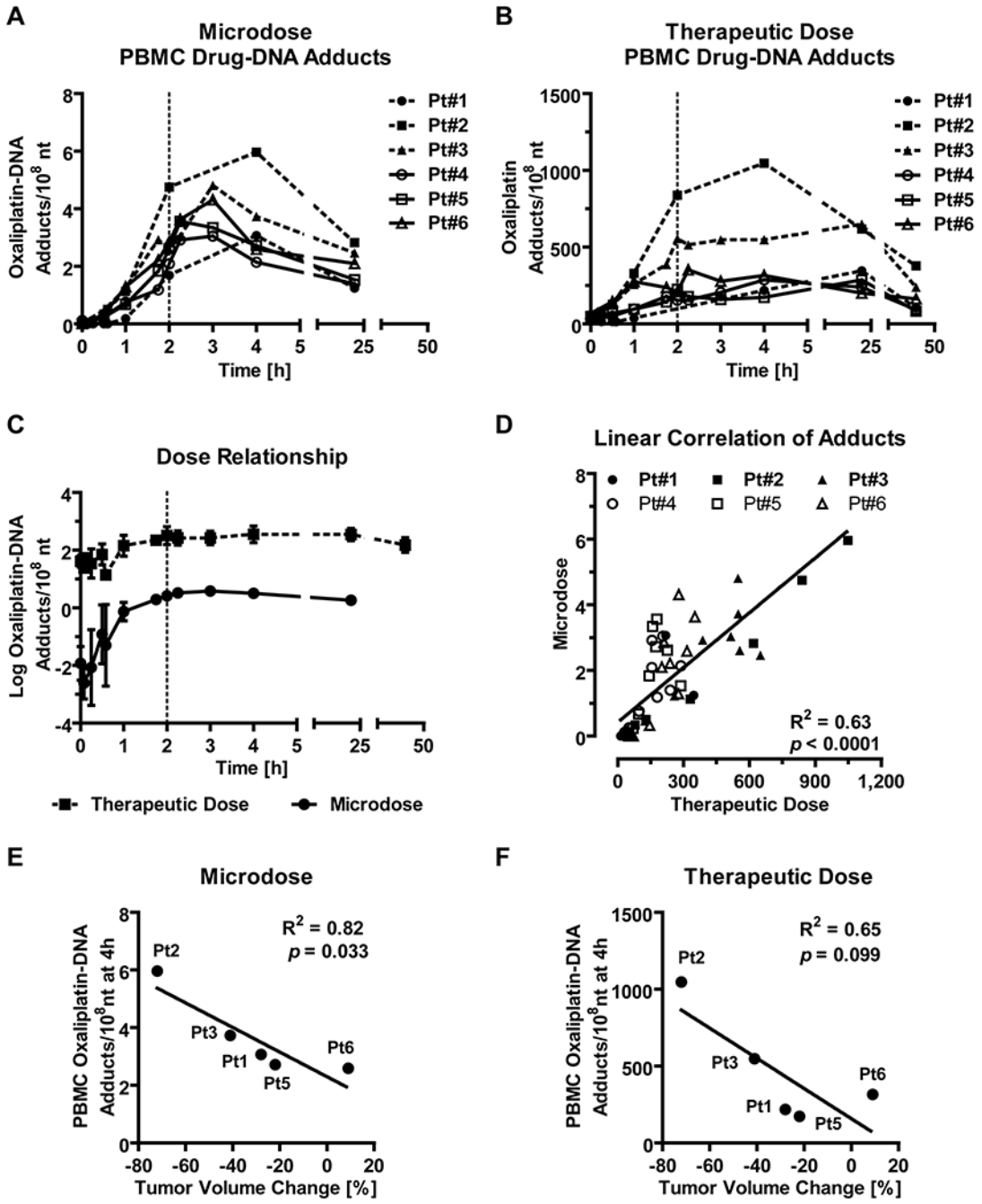

Figure 5. Microdose and therapeutic dose induced oxaliplatin-DNA adduct level are dose proportional and correlate with FOLFOX induced tumor reduction.

Indicated three CRC patients (patient #1: Pt1 ●, #2: Pt2 ■, and #3: Pt3 ▲) were infused for 2 h with **A)** a microdose or **B)** therapeutic dose of oxaliplatin. Oxaliplatin-DNA adduct levels were analyzed over a period of 24 or 48h via AMS. **C)** Dose proportionality of log microdose and therapeutic dose induced oxaliplatin-DNA adduct level (Mean values and standard error are shown). **D**) Linear correlation of oxaliplatin-DNA adduct level induced by a diagnostic micro- or a therapeutic dose of oxaliplatin ($R^2 = 0.63$, $p < 0.0001$). Correlation

of **E)** microdose ($R^2 = 0.82$, $p = 0.033$) or **F)** therapeutic dose ($R^2 = 0.65$, $p = 0.099$) induced oxaliplatin-DNA adduct level with mean tumor volume change after patient received 3-4 cycles of FOLFOX chemotherapy.

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Table 1:

Relevant cell line data for oxaliplatin IC_{50}, microdose (micro) and therapeutic dose (thera) induced oxaliplatin-DNA adduct level after 4 and 24h, area under the adduct curves (AUC adduct) induced by repair rates in four CRC cell lines.

Cell line	Oxaliplatin IC_{50} (μM)	DNA Damage: Oxaliplatin Adducts/10^8 nt				AUC Adducts/10^8 nt per hour		8-24h Repair Adducts/10^8 nt per hour	
		Thera		Micro		Thera	Micro	Thera	Micro
		4h	24h	4h	24h				
CLL-228	102	63134	3051	66.10	25.67	111,114	945.1	197	1.39
CLL-229	17.8	14651	8159	129.6	68.82	253,169	2180	302	2.66
CRL-2134	0.9	9640	5239	96.66	36.54	161,558	1414	173	2.13
HTB-38	0.3	69952	47821	851.8	312.1	1,220,000	9769	523	6.53

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Table 2:

Pharmacokinetic, pharmacodynamic and response data for three colorectal cancer patients given [^{14}C]oxaliplatin (Ox) as a microdose (Micro) or therapeutic dose (Thera) as part of the FOLFOX chemotherapy regimen. Response was determined according to RECIST criteria as either partial response (PR) or one almost complete response (CR) or progressive disease (PD).

Pt#	Dose	2h peak serum ox: ng/mL (C_{max})	AUC serum ox: ng/mL per hour	Ox-DNA level: adducts/10^8 nt			AUC: adducts/10^8 nt per hour	Repair: adducts /10^8 nt per hour		Chemo Therapy Response after 3-4 cycles*
				in vivo		in vitro		*in vivo*	*in vitro*	
				4h	24h	24h				
1	Micro	12.4	68.47	3.068	1.242	-	48.83	−0.091	-	PR (−28%)
	Thera	1,175	9,732	217.8	345.8	-	11,453	+6.396	-	
2	Micro	15.7	80.02	5.963	2.824	-	102.0	−0.157	-	PR (−72%)
	Thera	1,736	10,519	1,047	617.8	-	31,228	−21.48	-	
3	Micro	11.5	86.36	3.727	2.460	4.888	72.58	−0.063	+0.059	PR (−41%)
	Thera	1,792	18,215	548.2	649.1	465.4	24,221	+5.045	−4.143	
4	Micro	13.4	108.9	2.140	1.400	1.472	42.34	−0.037	−0.033	Not evaluable
	Thera	1,142	11,313	287.1	238.8	258.0	9,872	−2.412	−1.453	
5	Micro	8.6	66.06	2.719	1.536	1.938	50.73	−0.059	−0.039	PR (−41%)
	Thera	973.9	7,550	172.8	286.3	121.9	9,568	+5.475	−2.544	
6	Micro	14.3	114.8	2.589	2.095	1.071	56.51	−0.025	−0.076	PD (+9%)
	Thera	1,254	15,725	315.2	200.0	305.7	10.513	−5.760	−0.476	

* numbers represent percent tumor shrinkage after last cycle.

Pt# = patient number