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Preclinical Study of Treatment Response in HCT-116 Cells and Xenografts with ^1H -decoupled ^{31}P MRS

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Abstract

The topoisomerase I inhibitor, irinotecan, and its active metabolite SN-38 have been shown to induce G₂/M cell cycle arrest without significant cell death in human colon carcinoma cells (HCT-116). Subsequent treatment of these G₂/M-arrested cells with the cyclin-dependent kinase inhibitor, flavopiridol, induced these cells to undergo apoptosis. The goal of this study was to develop a noninvasive metabolic biomarker for early tumor response and target inhibition of irinotecan followed by flavopiridol treatment in a longitudinal study. A total of eleven mice bearing HCT-116 xenografts were separated into two cohorts where one cohort was administered saline and the other treated with a sequential course of irinotecan followed by flavopiridol. Each mouse xenograft was longitudinally monitored with proton (^1H)-decoupled phosphorus (^{31}P) magnetic resonance spectroscopy (MRS) before and after treatment. A statistically significant decrease in phosphocholine ($p = 0.0004$) and inorganic phosphate ($p = 0.0103$) levels were observed in HCT-116 xenografts following treatment, which were evidenced within twenty-four hours of treatment completion. Also, a significant growth delay was found in treated xenografts. To discern the underlying mechanism for the treatment response of the xenografts, *in vitro* HCT-116 cell cultures were investigated with enzymatic assays, cell cycle analysis, and apoptotic assays. Flavopiridol had a direct effect on choline kinase as measured by a 67% reduction in the phosphorylation of choline to phosphocholine. Cells treated with SN-38 alone underwent $83 \pm 5\%$ G₂/M cell cycle arrest compared to untreated cells. In cells, flavopiridol alone induced $5 \pm 1\%$ apoptosis while the sequential treatment (SN-38 then flavopiridol) resulted in $39 \pm 10\%$ apoptosis. *In vivo* ^1H -decoupled ^{31}P MRS indirectly measures choline kinase activity. The decrease in phosphocholine may be a potential indicator of early tumor response to the sequential treatment of irinotecan followed by flavopiridol in noninvasive and/or longitudinal studies.

Keywords

irinotecan; flavopiridol; choline kinase; colon cancer; ^1H -decoupled ^{31}P MRS; apoptosis

INTRODUCTION

The introduction of combination treatments with novel mechanism of action has led to considerable changes in the management of colon cancer. The treatment of human colon carcinoma with irinotecan followed by flavopiridol has demonstrated favorable results in humans (1) and a xenograft model (2). The success of such treatment regimens can be critically dependent on the timing or scheduled treatments of the two drugs. As this treatment development progresses and is translated into the clinic, there is a crucial need for robust tools and advanced techniques to measure the efficacy of such treatment regimes *in vivo* and to continuously monitor the tumor response to treatment. Early detection of failed treatment will constitute an opportunity to readjust the treatment or advance to a therapy that will improve the outcome of patients.

Previous studies have utilized ^1H magnetic resonance spectroscopy (MRS) to quantify apoptotic cell death (3) and ^{31}P MRS to monitor the effect of a choline kinase inhibitor on cells and tumor extracts (4). Scalar or spin-spin coupling, between ^1H and ^{31}P nuclei, results in a splitting of the observed signal into characteristic multiplet pattern visible in the ^{31}P MRS spectrum, although not resolvable *in vivo*. The spectral linewidth of such coupling between ^1H and ^{31}P nuclei in phosphomonoesters and phosphodiester is on the order of 5–10 Hz (5), which is greater than the achievable linewidth of *in vivo* ^{31}P MRS. Effective decoupling schemes can be used to collapse the multiplet into a singlet thereby increasing the signal-to-noise ratio (SNR) and simplifying the phosphomonoester resonance to separate phosphocholine and phosphoethanolamine peaks. Broadband multiple composite pulse decoupling sequences such as a MLEV-16 scheme (6–9) can be used to improve the spectral resolution and SNR of ^{31}P MRS spectra acquired during *in vivo* spectroscopy study. For *ex vivo* measurements, high resolution-magic angle spinning ^1H MRS (HR-MAS ^1H MRS) (10) is superior to static ^1H MRS because it averages out the susceptibility inhomogeneity across heterogeneous tissue samples (11) and eliminates the residual dipolar interactions of neighboring protons. As a result, the sensitivity and resolution of the spectra are significantly increased over static spectra, enabling a more accurate quantitative analysis of the biochemical properties of the sample (10,11).

Several alterations in cancer phospholipid metabolism have been previously noted (12–16). Disruption of the cytidinediphosphatecholine (CDP-choline) pathway, for example through depletion of its products, has been linked to apoptosis in a variety of cell systems (17–21). Phosphatidylcholine (PTC) is the most abundant structural component of cell membranes (22–24) and *de novo* synthesis of PTC is required for cell growth and differentiation. In mammalian cells, synthesis of PTC is mostly governed by the CDP-choline pathway (25). Choline is initially converted to phosphocholine (PC) by choline kinase (CK), and then to CDP-choline by cholinecitidyltransferase (CT). The final step in which CDP-choline is converted to PTC by cholinephosphotransferase (CPT) occurs very rapidly and results in minimal accumulation of the intermediary product, CDP-choline (26).

Experimental studies have shown that tumors with mutation in the *ras* oncogene and cell lines transformed with *ras* have an increase in CK activity and consequently have elevated levels of PC (27,28). Inhibition of CK, the initial enzyme in CDP-choline pathway, has been shown to inhibit proliferation of transformed cells and the growth of human colon tumor xenografts (27,29). It is known that the human colon cancer cell line, HCT-116, harbors the *ras* mutation (30). These findings are the impetus to investigate the use of *in vivo* marker of tumors with *ras* mutation and elevated choline kinase activity. Since phosphocholine is a product of choline kinase detectable by ^1H -decoupled ^{31}P MRS, this technique can be used to investigate the potential inhibition of choline kinase by flavopiridol *in vivo*. Treating HCT-116 cells with irinotecan or its active metabolite SN-38 induces G₂/M cell cycle arrest

with minimal cell death (2,31). Apoptosis in these G₂/M-arrested cells was enhanced after subsequent treatment with flavopiridol, a cyclin dependent kinase (CDK) inhibitor (2,31). However, the underlying molecular mechanism is not fully understood and has become an area of active research.

In this study, we hypothesized that the enhancement of apoptosis by flavopiridol in G₂/M-arrested HCT-116 cells, harboring the *ras* oncogene, may be due to the disruption of the CDP-choline pathway, specifically through the inhibition of CK. It would be ideal to develop noninvasive techniques to assess alterations in tumor cell biology, including the induction of apoptosis following a specific cancer therapy. Therefore, we elected to develop and validate a MRS technique that can be used to investigate CK and CT activity through the measurement of PC/choline and PTC/PC ratios, respectively, to evaluate the inhibitory effects of the drugs that target these critical steps in the CDP-choline pathway. As such, *in vivo* ¹H-decoupled ³¹P MRS and *in vitro* HR-MAS ¹H MRS are proposed as effective techniques for monitoring changes in the major components of the CDP-choline pathway.

EXPERIMENTAL

In vivo ³¹P MRS of HCT-116 tumor xenografts

All procedures with animals were performed according to the Memorial Sloan-Kettering Cancer Center's institutional guidelines for use and care of laboratory animals. Athymic nu/nu female mice (NCI, Bethesda, MD, 8–10 weeks of age) were implanted subcutaneously in the flank with 0.2 mL of HCT-116 cancer cell suspension (2.5×10⁶ cells/mL) in a 50:50 mixture of phosphate-buffered saline and matrigel (BD Bioscience, Franklin Lakes, NJ). During tumor growth, the tumor volume was determined by measuring the three orthogonal axes (*a*, *b*, *c*) of each tumor with calipers and by implementing the formula: $(\pi/6) \times a \times b \times c$ (32). A tumor volume of 150 mm³ to 200 mm³ was considered appropriate for *in vivo* MRS experiment. Data from longitudinal tumor volume measurements were fitted to an exponential growth rate, $y = y_0 \times e^{(kt)}$, where *y* is the tumor volume (mm³), *y*₀ is the measured volume at the start of MRS experiment, *k* is the rate constant, and *t* is the time (day). Tumor doubling time was calculated as 0.69/*k*. A more sophisticated model that takes into account the pharmacokinetics and pharmacodynamics of the drugs of HCT-116 xenograft after the sequential treatment of irinotecan followed by flavopiridol in mice was recently published (33).

Baseline MRS experiments were performed on each mouse prior to treatment. A cohort of mice (*N* = 7) was treated sequentially with a combination of irinotecan (100 mg/kg, *i.p.*) followed by flavopiridol (3 mg/kg, *i.p.*) with an interval of seven hours. Longitudinal MRS of the tumor within the same mouse was performed on the 1st day, 2nd day, and 7th day after the treatment. An equal volume of saline was administered to another cohort of mice (*N* = 4) as controls. All MRS experiments were carried out on a 7 Tesla Avance II spectrometer (Bruker BioSpin MRI GmbH, Ettlingen, Germany) for small animals and equipped with RF-channels operating at ¹H/³¹P Larmor frequencies of 300/121 MHz, respectively. The magnet is equipped with triple-axis gradients of up to 4 mT/m with an internal diameter of approximately 12 cm. The probe was a dual uncoupled-coil design with a three-turn parallel solenoidal coil as the phosphorus coil inside of a Helmholtz coil as the proton coil, and each coil was mounted on a separate LCR circuit (Figure 1). Images of the dual ¹H/³¹P probes are shown (Figure 1A–B) co-mounted unto a platform of a mouse stereotaxic-holder (10.5 cm in diameter) that was custom-built and is compatible with the magnet. The holder had a doubled walled glass-bowl with barbed fittings to accommodate a water heating-cooling system. Experiments were performed on mice (24±2 g) anesthetized with 2% isoflurane in oxygen at a flow rate of 1.5 L/min, and body temperature (37.2±1.0°C) was regulated with the water heating-cooling system. The mouse flank tumor was positioned in the custom-

built $^1\text{H}/^{31}\text{P}$ MR probe, secured with a suture, and placed in a water bath to reduce B_0 inhomogeneity (34). After the experimental setup in the 7T magnet was completed, each probe was properly tuned and matched to the Larmor frequency (f_0) of the respective nucleus (^1H or ^{31}P). The quality factor (Q) of each probe was calculated using the expression $Q=f_0/\Delta f$, where Δf is the bandwidth (full width at half maximum) of the resonant LCR circuit (Figure 1C–D). Typically, the Q 's for the ^1H (Figure 1C) and ^{31}P (Figure 1D) probes in the water bath were 33 ± 3 and 63 ± 2 , respectively.

Proton-decoupled one dimensional ^{31}P chemical shift imaging (1D ^{31}P CSI) was performed to measure metabolite signals of the tumor tissue without contributions from the mouse body. The 1D CSI phosphorus acquisition involved a FID-type sequence with a rectangular pulse, power of 25 dB, a 45° flip angle, 64 averages, TR of 1.8 s, acquisition size of 1024 points, spectral width of 10080 Hz, phase encode gradient duration of 0.4 ms, and a slice thickness of 4 mm. During each experiment, a calibration of the flip angle was performed with constant power while modulating the pulse length over a wide range of pulse lengths from 10 μs to 310 μs to determine an accurate 45° flip angle. Optimized decoupling was performed by modulating the decoupling transmitter power with a decoupling offset of 4.8 ppm from the resonance frequency of protons in water (see Supplementary Material). A composite pulse decoupling was implemented with MLEV-16 sequence of cyclic permutations in the supercycle (6–9). A 60 μl sphere of 50 mM methylenediphosphonic acid (Sigma Chemical Co., St. Louis, MO) doped with 10 μM MAGNEVIST®, gadopentetate dimeglumine, (Bayer Healthcare Pharmaceuticals, Germany) was mounted in a fixed position for chemical shift and pulse length calibrations as well a relative quantification of *in vivo* ^{31}P metabolite signals. The post-processing and quantification of the *in vivo* ^{31}P CSI data was performed with the XsOsNMR software package (kindly provided by Dr. D.C. Shungu and Mrs. X. Mao, Hatch MR Research Center, Columbia University College of Physicians and Surgeons, New York, NY).

Statistical analysis was performed using GraphPad Prism version 4.0a for Mac OS X (GraphPad Software, San Diego, California USA). Paired student's *t* test was performed to compare groups within an experiment. A *p*-value less than 0.05 was considered statistically significant.

In vitro 1D ^1H MAS MR analysis of HCT-116 cells

HCT-116 cells were cultured for 48–72 h to 60% confluence (first log phase) in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/mL penicillin and 100 mg/mL streptomycin, and maintained at 37°C in a humidified incubator with 5% carbon dioxide mixed in air. All cultures tested negative for *Mycoplasma* species. A stock solution of 5 mM SN-38 (Pharmacia, Peapack, NJ) was prepared in DMSO. A flavopiridol (National Cancer Institute, Bethesda, MD) stock solution of 4.5 mM was prepared in water and stored at -20°C . Cell cultures were exposed to drug free medium ($N=4$), 20 nM of SN-38 treatment alone ($N=4$), 150 nM of flavopiridol treatment alone ($N=4$), or to 20 nM of SN-38 followed by 150 nM flavopiridol after 24 h interval ($N=4$). Cell cultures were trypsinized, harvested, counted with a hemacytometer, and 8×10^6 cells were resuspended in PBS/D $_2$ O (pH=7.4). HCT-116 cells (8×10^6 cells) were washed twice with PBS/D $_2$ O and then placed in a 4-mm-O.D. zirconium rotor for MR analysis. Five μL of 95 mM 3-(trimethylsilyl) propionic 2,2,3,3-d $_4$ acid, sodium salt (TSP) (Cambridge Isotope Laboratories, Inc., Andover, MA) added to the rotor served as internal chemical shift and concentration standard. All spectra were obtained on a Bruker Avance 600 MHz at 20°C using a Bruker 4-mm high-resolution $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ MAS probe. A spinning rate of 5 kHz was used for all experiments. Spectra for each sample were acquired using a $\pi/2$ pulse preceded by 2 s water suppression pulse, with accumulation of 128 transients, a repetition delay of 10 seconds, and a spectral

width of 7200 Hz. Data were processed with 1 Hz line broadening followed by Fourier transformation. Assignments of metabolites resonance peaks were made by comparing chemical shifts to those of standard compounds measured in PBS/D₂O (pH=7.4), and were also based on previously published NMR resonances (35,36). Cellular metabolites were quantified by computing the integral of assigned resonances using Lorentzian line-shapes to fit the 1D ¹H spectra. In spectral regions of overlapping resonances, deconvolution was applied using the NMR post-processing software 1D WINNMR 6.1 software (Bruker, Ettlingen, Germany). Peak integrals of metabolites were tabulated, and the ratio of PC/choline was used as an indirect measure of CK activity where as the ratio of PTC/PC was used as an indirect measure of CT activity.

Statistical analysis was performed using GraphPad Prism version 4.0a for Mac OS X (GraphPad Software, San Diego, California, USA). A two-tailed unpaired student's t test was performed to verify the statistical significance of the means in the compared groups. A p-value less than 0.05 was considered statistically significant. All results are displayed as mean±SEM (standard error of mean) of measures in groups.

Measurement of the effect of flavopiridol on purified choline kinase [EC 2.7.1.32]

To determine if the inhibition of choline kinase (CK) by flavopiridol was due to a direct effect, purified yeast CK [EC 2.7.1.32] from *Saccharomyces cerevisiae* was examined by solution proton MR spectroscopy. The enzymatic reaction from choline to phosphocholine was carried out in a buffer solution containing 10 mM tris acetate, 13 mM β-mercaptoethanol, 1 mM EDTA and 15 mM magnesium chloride at pH 7.2. A total of 0.2 units/mL purified CK [EC 2.7.1.32] was used with the final concentrations of 2.87 mM choline and 0.86 mM ATP for each experiment (N=4). All reagents were obtained from Sigma Aldrich, St. Louis MO, and dissolved in 95% distilled H₂O/5% D₂O. Flavopiridol was diluted directly from a 4.5 mM stock solution into the solution for the enzymatic reaction to final concentrations of 0.5 μM and 1.0 μM. The final solutions were transferred to a standard 5 mm liquid NMR tube, the mixture was vortexed vigorously, and placed immediately into the MR spectrometer. Five μL of a 95 mM TSP solution was used in each experiment as an internal chemical shift and concentration standard. Data acquisition was performed on a Bruker Avance 600 MHz high-resolution MR spectrometer at 20°C using Bruker 5-mm TXI liquid probe. Water suppression was achieved with a 2 s pre-saturation pulse. Spectra were obtained every 100 s and averaged over 600 seconds (10 minutes). Spectral analysis was performed using the 1D WINNMR 6.1 software (Bruker, Ettlingen, Germany). Phosphocholine levels were determined from the ¹H MR spectra, as described in the previous section, and plotted with respect to time. Linear regression analysis was performed on the data sets to compute the slope or production rate, compute the goodness-of-fit (r²), and compare the slopes or production rate of various flavopiridol treatments. Statistical analysis was performed using GraphPad Prism version 4.0a for Mac OS X (GraphPad Software, San Diego, California USA).

Cell Cycle Analysis

HCT-116 cells were cultured for 48–72 h to 60% confluence (first log phase) and exposed to drug free medium (N=3), 20 nM of SN-38 treatment alone (N=4), 150 nM of flavopiridol treatment alone (N=4), or in combinations (N=4) with 24 h interval. At the conclusion of treatment, the medium from the flasks was collected to ensure capture of floating cells, cell cultures were trypsinized, and segregated into replicates for further analysis. The cell cycle distribution was analyzed by flow cytometry as previously described (37). Briefly, cells were fixed in ice cold 70% ethanol. Following fixation, cell pellets were washed twice with PBS containing 0.05% tween-20 and then suspended in 5 μg/mL of propidium iodide (Calbiochem, San Diego, CA) containing 1 μL/mL of RNase. Cell cycle distribution was

determined following propidium iodide staining using a FACS Calibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA). The level of apoptosis was quantified by measuring the amount of subdiploid (sub-G₁-G₀) cells stained with propidium iodide.

Statistical analysis was performed using GraphPad Prism version 4.0a for Mac OS X (GraphPad Software, San Diego, California, USA). A two-tailed unpaired student's t test was performed to verify the statistical significance of the means in the compared groups. A p-value less than 0.05 was considered statistically significant. All results are displayed as mean percent±SEM (standard error of mean) of measures in groups.

RESULTS

In vivo ¹H-decoupled ³¹P spectroscopy indirectly measures choline kinase activity

Representative ³¹P MR spectra of one xenograft at baseline and one day after combined irinotecan followed by flavopiridol treatment are shown in Figure 2. There is notable reduction in the phosphocholine (PC) and inorganic phosphorus (P_i) resonance peaks in response to treatment. The split of the inorganic phosphorus (P_i) resonance peak may suggest changes in intracellular pH (38–40) and may reflect two distinct extracellular (P_i-left, low field) and intracellular (P_i-right, up field) compartments (38,40,41). However, we did not investigate the underlying mechanism to these pH changes. In general, the intracellular compartments of tumors exhibit a neutral or slightly alkaline pH (42). The pretreated baseline spectrum, which indicates the split P_i resonance peak is resolved into two resonances, coalesces to a single resonance peak after treatment. It is possible that in the present study, modulation of the P_i resonance peak may be due to the drug induced changes culminating into a decrease in intracellular pH levels or binding of P_i.

No significant change in phosphoethanolamine levels was found in either the combined treatment cohort (0.90±0.09, N=7, *p*=0.33) or the saline-treated cohort (1.07±0.24, N=4, *p*=0.80) as compared to unit baseline levels (Figure 3A). A statistically significant drop in phosphocholine (0.49±0.07, N=7, *p*=0.0004) and inorganic phosphate (0.65±0.10, N=7, *p*=0.0103) levels were observed in HCT-116 xenografts one day after combined irinotecan followed by flavopiridol treatment as compared to unit baseline levels (Figure 3B–C). No significant difference was found in phosphocholine (0.84±0.07, N=4, *p*=0.11) and inorganic phosphate (1.52±0.32, N=4, *p*=0.20) levels for saline treatment as compared to unit baseline levels (Figure 3B–C). The tumor growth rates of the two cohorts are shown in Figure 3D along with the 95% confidence interval band for the exponential fit with nonlinear regression. Tumor doubling time in mice treated with irinotecan followed by flavopiridol was significantly longer (54.81±0.01 days, *r*²=0.42) than in the saline-treated cohort (6.86±0.02 days, *r*²=0.91). A more sophisticated model that takes into account the pharmacokinetics and pharmacodynamics of both drugs (33) also revealed a substantial growth delay of HCT-116 xenograft after the sequential treatment of irinotecan followed by flavopiridol in mice.

Indirect choline kinase (CK) and cholinecitidyltransferase (CT) activities measured with 1D ¹H MAS MR analysis

Representative HR-MAS ¹H MR spectra of HCT-116 cells are shown in Figure 4, with an insert of a zoomed region from 2.5 ppm to 3.5 ppm (Figure 4E). The ratio of PC/choline was used as an indirect measure of CK activity in HCT-116 cells (Figure 5). The intervals between the combined treatments of SN-38 followed by flavopiridol showed significantly smaller PC/choline ratio at intervals of 18 h (14.00±0.94, N=4, *p*=0.0171) and 24 h (7.50±1.50, N=4, *p*=0.0076) as compared to the group without a time interval (43.90±9.10, N=4) in the combined treatment (Figure 5A). In another set of HCT-116 cells experiments

(Figure 5B), the combined SN-38 followed by flavopiridol treatment with 24 h interval (9.23 ± 1.54 , $N=4$, $p=0.0022$) or flavopiridol treatment alone (16.54 ± 2.69 , $N=4$, $p=0.0344$) significantly reduced PC/choline ratio as compared to drug free medium exposure (28.46 ± 3.45 , $N=4$). The PC/choline ratio of SN-38 treatment alone (41.54 ± 5.77 , $N=4$, $p=0.0997$) showed no significant difference than in drug free medium exposure (28.46 ± 3.45 , $N=4$). The treatment with flavopiridol alone (16.54 ± 2.69 , $N=4$, $p=0.0077$) or in combination with SN-38 (9.23 ± 1.54 , $N=4$, $p=0.0016$) significantly reduced PC/choline ratio as compared to SN-38 treatment alone (41.54 ± 5.77 , $N=4$). There was no significant difference between flavopiridol treatment alone (16.54 ± 2.69 , $N=4$, $p=0.0564$) as compared to combined SN-38 followed by flavopiridol treatment with 24 h interval (9.23 ± 1.54 , $N=4$). The results herein may suggest that a flavopiridol treatment alone or in combination with SN-38 significantly reduces CK activity, and that a time interval between administration of SN-38 and favopiridol may be necessary to potentiate its effect on CK activity.

The ratio of PTC/PC was used as an indirect measure of CT activity in HCT-116 cells (Figure 6). The results indicate that there is no significant difference in CT activity for flavopiridol treatment alone (1.72 ± 0.24 , $N=6$, $p=0.6057$) or SN-38 treatment alone (1.14 ± 0.18 , $N=6$, $p=0.2064$) as compared to drug free medium exposure (1.54 ± 0.23 , $N=6$). Also, there is no significant difference in PTC/PC ratio between drug free medium exposure (1.54 ± 0.23 , $N=6$) and the combined treatment of SN-38 followed by flavopiridol (2.56 ± 0.40 , $N=6$, $p=0.0524$).

Flavopiridol directly inhibits purified choline kinase [EC 2.7.1.32]

Phosphocholine levels were plotted with respect to time for no flavopiridol, 0.5 μM flavopiridol, and 1.0 μM flavopiridol treatments (Figure 7). The production rate or slope of phosphocholine in response to 1.0 μM flavopiridol treatment ($1.46 \pm 0.15 \times 10^{-3}$ rel.u./min; $r^2=0.90$) was significantly lower than in response to 0.5 μM flavopiridol treatment ($3.25 \pm 0.22 \times 10^{-3}$ rel.u./min; $r^2=0.94$, $p<0.0001$), and in the untreated control ($9.33 \pm 0.51 \times 10^{-3}$ rel.u./min; $r^2=0.97$, $p<0.0001$). In addition, the runs test was used to determine if each data set fits a straight line (significantly nonlinear). If the overall production rates or slopes were identical, there is less than a 0.01% chance of randomly choosing data points with slopes this different. Henceforth, we can conclude that the differences between the slopes are extremely significant. This finding suggests that flavopiridol directly inhibits choline kinase activity, and it is known (43) that flavopiridol competitively inhibits CDKs by directly binding to their ATP binding site.

Subsequent SN-38 and flavopiridol treatment induces apoptosis

The cell cycle distribution and apoptotic fraction for HCT-116 cells culture exposed to SN-38 alone ([T2], $N=4$), flavopiridol alone ([T3], $N=4$), or the combination of SN-38 followed by flavopiridol ([T4], $N=4$) are presented in Figure 8. The percent of apoptotic cells from combined SN-38 followed by flavopiridol treatment ($39 \pm 10\%$, $N=4$) was significantly higher than for SN-38 treatment alone ($1.00 \pm 0.01\%$, $N=4$, $p=0.009$) or flavopiridol treatment alone ($5 \pm 1\%$, $N=4$, $p=0.015$). The fraction of G₂/M arrested cells for SN-38 treatment alone ($83 \pm 5\%$, $N=4$) was significantly higher than for flavopiridol treatment alone ($25 \pm 1\%$, $N=4$, $p<0.0001$), but not significantly different when compared to combined SN-38 followed by flavopiridol treatment ($76 \pm 5\%$, $N=4$, $p=0.360$). The apoptotic and G₂/M fractions of cells exposed to drug free medium were 0.85 ± 0.15 ($N=3$) and 19 ± 2 ($N=3$), respectively. These results indicate that SN-38 facilitates G₂/M cell arrest, and follow-up treatment with flavopiridol induces these G₂/M-arrested cells to synergistically undergo apoptosis.

DISCUSSION

The results of this study suggest the techniques of ^1H -decoupled ^{31}P and HR-MAS ^1H MRS can be used to investigate the mechanisms of action of agents that target the key enzymes in the CDP-choline pathway and for assessing apoptosis. A statistically significant decrease in phosphocholine and inorganic phosphate levels were observed in HCT-116 xenografts following combined treatment, which were evidenced within twenty-four hours of treatment completion. A previous report has shown statistically significant growth delays when p21-intact HCT-116 xenografts were treated sequentially with irinotecan and flavopiridol (2). Consistent with this result, we found that a single treatment with irinotecan followed by flavopiridol reduced the doubling time of tumors within a week after treatment. We performed a pilot study (*see* Supplementary Material) on mice bearing HCT-116 xenograft in cohorts treated with irinotecan alone (N=3), flavopiridol alone (N=3), and in combination as irinotecan followed by flavopiridol (N=3). Our assessment assured no considerable difference was depicted in the baseline ^{31}P MRS spectra compared with that obtained 24 hours after independent treatment with either irinotecan alone or flavopiridol alone. Our pilot data is congruent with previous published work, which showed that flavopiridol treatment alone had no significant effect on HCT-116 xenografts growth (2). Also, a phase II trial of flavopiridol alone in patients with advanced colorectal cancer did not show significant effect (44). Thus, we focused on the combined treatment for the *in vivo* studies, and we performed the independent and combined treatment regimens *in vitro* to elucidate the mechanism of such treatment on choline metabolism. Ultimately, our objective was to investigate a detectable *in vivo* marker for early treatment response of irinotecan followed by flavopiridol, which is the preferred treatment in the clinic rather than either drug alone (1). The 7 h interval in our preclinical study for this sequential treatment of irinotecan followed by flavopiridol was empirically selected based on experiments from our previous study (2). *In vitro* HCT-116 cell experiments have shown significant treatment effect at longer intervals (18–24 h) between the sequential administration of the two drugs than in the *in vivo* studies (2,31). Taking into account the pharmacokinetics and pharmacodynamics of the drugs *in vivo*, a recent study has modeled an optimal period of 8–10 h between doses, which compares favorably with the 7 h interval used in our study (33).

To discern the underlying mechanism for this effect in the xenografts, *in vitro* HCT-116 cell cultures were investigated with enzymatic assays, cell cycle analysis, and apoptotic assays. The ratio of PC/choline was used as an indirect measure of choline kinase (CK) activity whereas the ratio of PTC/PC was used as an indirect measure of cholinecetyltransferase (CT) activity. Our study suggests that flavopiridol alone or preceded by treatment with SN-38 or irinotecan significantly reduces CK activity, and that the time interval between the administration of the two drugs plays a pivotal role in potentiating the treatment response of CK activity. On the other hand, there was no significant difference in CT activity for flavopiridol treatment alone or SN-38 treatment alone when compared to untreated controls. The significantly reduced production rate of phosphocholine by purified CK [EC 2.7.1.32] in response to flavopiridol treatment suggests that flavopiridol directly inhibits CK activity, and it is known that flavopiridol competitively inhibits CDKs by directly binding to their ATP binding site (43,45). In addition, the fraction of G₂/M arrested cells was significantly higher in the cohort treated with SN-38 alone than in other cohorts. The percent of apoptotic cells was significantly higher in the cohort treated with SN-38 followed by flavopiridol than in other cohorts. These results indicate that SN-38 facilitates G₂/M cell arrest in HCT-116 cells, and that follow-up treatment with flavopiridol induces these G₂/M-arrested cells to undergo apoptosis, which is in congruence with precedent reports (2,31).

The topoisomerase I inhibitor, irinotecan, and its active metabolite SN-38 have been shown to induce G₂/M cell cycle arrest without significant cell death in human colon carcinoma

cells (HCT-116) (2,31). Subsequent treatment of these G₂/M-arrested cells with flavopiridol induced these cells to undergo apoptosis. Flavopiridol inhibits a wide range of phosphokinases including the cyclin-dependent kinases as well as tyrosine and serine kinases (45). The potent inhibitory effects of flavopiridol are mediated largely through the inhibition of CDKs in cycling cells (45) although flavopiridol is also capable of inducing cell death in non-cycling cells (46). Furthermore, flavopiridol is known to transcriptionally down regulate many anti-apoptotic proteins (47,48). Flavopiridol is able to induce apoptosis in a wide variety of tumor cells *in vitro* and *in vivo*, however the percentage of cells undergoing apoptosis varies considerably, and in some systems, may be independent of cyclin-dependent kinase activity (45). These findings may suggest that there are additional mechanisms of action. Our experiments identified CK to be an additional targeted kinase among the many phosphokinases inhibited by flavopiridol. Flavopiridol has been shown to inhibit CDKs by directly binding to the ATP-binding site (43) which lead us to hypothesize that flavopiridol may inhibit CK in a similar manner. In support of this observation, high-resolution proton MR methods were used to quantify PC synthesis rates in a purified solution of yeast CK, which contained constant concentrations of CK and ATP. The addition of 0.5 μM flavopiridol results in a 65% decrease in the PC synthesis rate. Increasing the flavopiridol concentration to 1.0 μM resulted in an 84% decrease in the PC conversion rate. This suggests that flavopiridol is directly inhibiting CK through a mechanism similar to the inhibition of CDKs. The IC₅₀ of less than 0.5 μM for flavopiridol in isolated yeast CK is well within the 500 nM ranges that are readily achievable in humans and is similar to the IC₅₀ found for flavopiridol for CDKs and in fact much lower than the IC₅₀ for EGF receptor kinases, pp60 Src, PKC and Erk-1 kinases (45).

Collectively, this study demonstrates the usefulness of *in vivo* ¹H-decoupled ³¹P MRS and HR-MAS ¹H MRS to evaluate drug-induced metabolic changes in the CDP-choline pathway, which may serve as surrogate marker of tumor cell apoptosis, and tumor regression. This study suggests that flavopiridol potentiates apoptosis in HCT-116 cells by potentially inhibiting CK as one possible mechanism. *In vivo* ¹H-decoupled ³¹P MRS indirectly measures changes in choline kinase activity and the observed decrease in phosphocholine may be a potential indicator of early tumor response to the sequential treatment of irinotecan followed by flavopiridol in a noninvasive and/or longitudinal study. In light of these results, plans are underway to test whether these MRS changes can be observed clinically in patients treated with sequential irinotecan and flavopiridol therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

¹H MRS proton magnetic resonance spectroscopy

³¹P MRS	phosphorus magnetic resonance spectroscopy
SNR	signal-to-noise ratio
MLEV	Malcolm LEVitt sequences
CDP-choline	cytidinediphosphatecholine
PTC	phosphatidylcholine
PC	phosphocholine
CK	choline kinase
CT	cholinescitidyltransferase
CPT	cholinephosphotransferase
HCT-116	human colon cancer cells
SN-38	active metabolite of irinotecan
NMR	nuclear magnetic resonance
RF	radiofrequency
LCR	inductance capacitance resistance
1D CSI	one dimensional chemical shift imaging
FID	free induction decay
RPMI	Roswell Park Memorial Institute
DMSO	dimethyl sulfoxide
TSP	3-(trimethylsilyl) propionic 2,2,3,3-d ₄ acid sodium salt
EDTA	ethylenediaminetetraacetic acid
EC	enzyme commission
PBS	phosphate buffered saline
FACS	fluorescence activated cell sorting
DAG	diacylglycerol
EGF	epidermal growth factor
PKC	protein kinase C
SEM	standard error of mean

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Figure 1. Photographs of the custom-built ^1H and ^{31}P coils [A] co-mounted on a mouse stereotaxic-holder holder [B] with a close-up view of the 3-turned parallel solenoidal coil as the ^{31}P coil inside the Helmholtz coil as the ^1H coil. A typical tuned and matched frequency of the resonant LCR circuit at experimental setup for the [C] ^1H coil and [D] ^{31}P coil in water bath.

Figure 2. Representation of *in vivo* ^1H -decoupled ^{31}P MR spectra of HCT-116 xenograft obtained longitudinally from [A] prior to treatment and [B] one day after sequential treatment of irinotecan (100 mg/kg i.p.) followed by flavopiridol (3 mg/kg i.p.). Spectral peaks are labeled as: **1**, methylenediphosphonic acid (MDP, 50 mM standard); **2**, phosphoethanolamine (PE); **3**, phosphocholine (PC); **4**, inorganic phosphate (P_i); **5**, phosphocreatine (PCr); **6**, gamma-nucleoside triphosphate (γ -NTP); **7**, alpha-nucleoside triphosphate (α -NTP); **8**, nicotinamide adenine dinucleotide (NAD/NADH); **9**, beta-nucleoside triphosphate (β -NTP), respectively. Also shown for each spectrum, are the spectra fits, the signals fitted (components), and residual spectrum after fitting are shown below each spectrum. Sequential treatment demonstrates a significant reduction in PC and P_i by the 1st day after treatment as compared to baseline PC.

In vivo HCT-116 xenograft results

Figure 3. Longitudinal results of *in vivo* ^1H -decoupled ^{31}P MR spectroscopy of HCT-116 xenografts: [A] phosphoethanolamine, [B] phosphocholine, [C] inorganic phosphate, and [D] tumor growth rate. Data points are displayed as mean $\pm$ SEM, and the p-values were evaluated by paired t test ($^{\text{ns}}$ p-value > 0.05 , * p-value ≤ 0.05 , ** p-value ≤ 0.01 , *** p-value ≤ 0.001).

Figure 4.

Representative high-resolution MAS ^1H MR spectra of HCT-116 cells exposed to 20 nM SN-38 for 24 hours and/or 150 nM flavopiridol for 24 hours where ND denote serum-free media and the numeric subscripts designate hours of treatment schedules: **[A]** drug free medium ($\text{ND}_{24} \rightarrow \text{ND}_{24}$); **[B]** SN-38 treatment alone ($\text{SN-38}_{24} \rightarrow \text{ND}_{24}$); **[C]** flavopiridol treatment alone ($\text{ND}_{24} \rightarrow \text{FL}_{24}$); **[D]** combined SN-38 followed by flavopiridol ($\text{SN-38}_{24} \rightarrow \text{FL}_{24}$); **[E]** a zoomed region from 2.6 to 3.5 ppm.

Figure 5.

Choline kinase (CK) activity indirectly determined *in vitro* by the MR measurement of phosphocholine to choline ratio in HCT-116 cells. **[A]** HCT-116 cells were exposed to 20 nM SN-38 for 24 hours followed by 150 nM flavopiridol at various time intervals as indicated (N=4, mean±SEM, relative units). **[B]** HCT-116 cells were exposed to drug free medium (ND₂₄→ND₂₄), SN-38 treatment alone (SN-38₂₄→ND₂₄), flavopiridol treatment alone (ND₂₄→FL₂₄) or combined SN-38 followed by flavopiridol (SN-38₂₄→FL₂₄). All bar graphs are displayed as mean±SEM, and the p-values were evaluated by two-tailed unpaired t test (^{ns}p-value>0.05, *p-value≤0.05, ** p-value≤0.01, *** p-value≤0.001).

cholinecitidyltransferase (drugs effect)

Figure 6.

Cholinecitidyltransferase (CT) activity indirectly determined *in vitro* by the MR measurement of phosphatidylcholine to phosphocholine ratio (PTC/PC) in HCT-116 cells exposed to drug free medium (ND₂₄→ND₂₄), SN-38 treatment alone (SN-38₂₄→ND₂₄), flavopiridol treatment alone (ND₂₄→FL₂₄), or SN-38 followed by flavopiridol (SN-38₂₄→FL₂₄). Box-and-Whiskers graph: the box extends from the 25th percentile to the 75th percentile with a line at the 50th percentile (the median), and the whiskers extend above and below the box to show the highest and lowest values, respectively. The PTC/PC ratio did not differ significantly between treatment groups, and the p-values were evaluated by two-tailed unpaired t test (^{ns}p-value>0.05).

Saccharomyces cerevisiae

Choline kinase (EC 2.7.1.32)

Figure 7. Indirect MR measurement of purified choline kinase [EC 2.1.7.32] activity from *Saccharomyces cerevisiae*. Phosphocholine levels were measured from ^1H MR spectra and plotted with respect to time. Linear regression analysis was performed on the data sets to compute the slope or production rate, the goodness-of-fit (r^2), and compare the slopes or production rate of various flavopiridol treatments.

Cell cycle analysis

Figure 8.

Cell cycle distribution and percent apoptosis of HCT-116 treated cells with different treatment schedules as drug free medium (ND₂₄→ND₂₄ [T1]), 20 nM SN-38 alone (SN-38₂₄→ND₂₄ [T2]), 150 nM flavopiridol alone (ND₂₄→FL₂₄ [T3]), or SN-38 followed by flavopiridol (SN-38₂₄→FL₂₄ [T4]) as determined by flow cytometry. ND represents serum-free media and subscripts designate hours of treatment. All bar graphs are displayed as mean percent±SEM (N=3–4), and the p-values were evaluated by two-tailed unpaired t test.