

Diverse mechanisms of growth inhibition by luteolin, resveratrol, and quercetin in MIA PaCa-2 cells: a comparative glucose tracer study with the fatty acid synthase inhibitor C75

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Abstract The rationale of this dose matching/dose escalating study was to compare a panel of flavonoids—luteolin, resveratrol, and quercetin—against the metabolite flux-controlling properties of a synthetic targeted fatty acid synthase inhibitor drug C75 on multiple macromolecule synthesis pathways in pancreatic tumor cells using [1,2-¹³C₂]-D-glucose as the single precursor metabolic tracer. MIA PaCa-2 pancreatic adenocarcinoma cells were cultured for 48 h in the presence of 0.1% DMSO (control), or 50 or 100 μM of each test compound, while intracellular glycogen, RNA ribose, palmitate and cholesterol as well as extra cellular ¹³CO₂, lactate and glutamate production patterns were measured using gas chromatography/mass spectrometry (GC/MS) and stable isotope-based dynamic

metabolic profiling (SiDMAP). The use of 50% [1,2-¹³C₂]-D-glucose as tracer resulted in an average of 24 excess ¹³CO₂ molecules for each 1,000 CO₂ molecule in the culture media, which was decreased by 29 and 33% ($P < 0.01$) with 100 μM C75 and luteolin treatments, respectively. Extracellular tracer glucose-derived ¹³C-labeled lactate fractions (Σm) were between 45.52 and 47.49% in all cultures with a molar ratio of 2.47% M + 1/ Σm lactate produced indirectly by direct oxidation of glucose in the pentose cycle in control cultures; treatment with 100 μM C75 and luteolin decreased this figure to 1.80 and 1.67%. The tracer glucose-derived ¹³C labeled fraction (Σm) of ribonucleotide ribose was 34.73% in controls, which was decreased to 20.58 and 8.45% with C75, 16.15 and 6.86% with luteolin, 27.66 and 19.25% with resveratrol, and 30.09 and 25.67% with quercetin, respectively. Luteolin effectively decreased nucleotide precursor synthesis pentose cycle flux primarily via the oxidative branch, where we observed a 41.74% flux (M + 1/ Σm) in control cells, in comparison with only a 37.19%, 32.74%, or a 26.57%, 25.47% M + 1/ Σm flux ($P < 0.001$) after 50 or 100 μM C75 or luteolin treatment. Intracellular de novo fatty acid palmitate (C16:0) synthesis was severely and equally blocked by C75 and luteolin treatments indicated by the 5.49% (control), 2.29 or 2.47% (C75) and 2.21 or 2.73% (luteolin) tracer glucose-derived ¹³C-labeled fractions, respectively. On the other hand there was a significant 192 and 159% ($P < 0.001$), and a 103 and 117% ($P < 0.01$) increase in tracer glucose-derived cholesterol after C75 or luteolin treatment. Only resveratrol and quercetin at 100 μM inhibited tracer glucose-derived glycogen labeling (Σm) and turnover by 34.8 and 23.8%, respectively. The flavonoid luteolin possesses equal efficacy to inhibit fatty acid palmitate de novo synthesis as well as nucleotide RNA ribose turnover via the oxidative

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branch of the pentose cycle in comparison with the targeted fatty acid synthase inhibitor synthetic compound C75. Luteolin is also effective in stringently controlling glucose entry and anaplerosis in the TCA cycle, while it promotes less glucose flux towards cholesterol synthesis than that of C75. In contrast, quercetin and resveratrol inhibit glycogen synthesis and turnover as their underlying mechanism of controlling tumor cell proliferation. Therefore the flavonoid luteolin controls fatty and nucleic acid syntheses as well as energy production with pharmacological strength, which can be explored as a non-toxic natural treatment modality for pancreatic cancer.

Keywords Metabolic profile · Phytochemicals · Pancreatic cancer · Fatty acid synthase · Lipogenesis · Luteolin · Resveratrol · Quercetin · C75

1 Introduction

Fatty acid synthase (FAS; EC 2.3.1.85) catalyzes the synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. Although acetyl-CoA carboxylase (ACC), the enzyme that carboxylates acetyl-CoA to malonyl-CoA, catalyzes the rate-limiting reaction in the fatty acid synthetic pathway, FAS is the key biosynthetic enzyme in the pathway. FAS has been shown to be particularly active in cancer cells, producing palmitate, which is the 16 carbon saturated fatty acid of cellular membranes. In highly lipogenic tissues such as liver, adipose tissue, and the lactating breast, the lipid synthetic pathway functions normally to store excess energy intake, synthesize lipid from carbohydrate or protein if the diet is low in fat, or produce lipids for milk production. However, in cancer cells endogenously synthesized fatty acids are esterified predominantly to phospholipids for membrane lipid synthesis, which promotes cell replication, rather than used for triglyceride energy storage. In fact, pharmacological and small interference RNA-mediated inhibition of FAS decreases the synthesis of phospholipids, suggesting that the high level of lipogenesis in cancer cells is primarily for the synthesis of membranes (Swinnen et al. 2006).

Since the identification of the previously described breast oncoprotein OA-519 as FAS (Kuhajda et al. 1994), overexpression of FAS has been measured in a broad spectrum of cancers, include that of prostate, ovary, colon, endometrium, lung, bladder, stomach, esophagus, tongue, oral cavity, kidney, and skin, as well as in mesotheliomas, retinoblastomas, and nephroblastomas (Kuhajda 2000). More recently FAS expression in pancreatic ductal adenocarcinoma has been reported, and is correlated with advanced tumor stage (Alo et al. 2007; Witkiewicz et al. 2008). Patients with FAS-positive breast, prostate, or

endometrial cancer have a poorer prognosis than those with low or absent FAS expression, and in breast cancer patients, high levels of FAS expression was correlated with shortened disease-free and overall survival (Alo et al. 1996; Jensen et al. 1995). Interestingly, FAS protein can be assayed in blood by ELISA, and elevated FAS levels have been identified in the sera of patients with breast, prostate, colon and ovarian cancers, indicating circulating FAS antigen levels may potentially be biomarkers of malignancy (Kuhajda 2006).

The functionality of the FAS protein is similar in both tumor and normal lipogenic tissues and the FAS protein catalyzes multiple enzymatic reactions. In particular, the β -ketoacyl synthase activity of the protein is a target for drug development. A synthetic, small-molecule inhibitor of FAS termed C75 (tetrahydro-4-methylene-2R-octyl-5-oxo-3S-furancarboxylic acid) has significant antitumor effects against human cancer cell lines in vitro, and against human breast, prostate, mesothelioma, and ovarian cancer xenografts (Kuhajda et al. 2000; Kuhajda 2006; Fig. 1). Inhibition of FAS by pharmacological inhibitors both in vitro and in vivo has been shown to induce apoptosis in breast and prostate cancer cells (Pizer et al. 1996, 2000, 2001; Zhou et al. 2003). This observation implies that cancer cells are dependent on the fatty acid synthesis pathway for survival; however the mechanisms linking FAS inhibition to apoptosis are not clear.

Certain phytochemicals (plant-derived bioactive compounds) are also inhibitors of FAS activity in vitro. A number of polyphenols have been surveyed for this effect, and at least 12 flavonoids that inhibit FAS enzyme activity with IC_{50} values ranging from 2 to 112 μ M were identified (Tian 2006). Based on this report we chose a set of flavonoids to explore further. Quercetin (3,3',4',5,7-pentahydroxyflavone) is widely available from many plant food sources, including from apple, onions, kale, broccoli, and French beans (Harris et al. 2005). Resveratrol

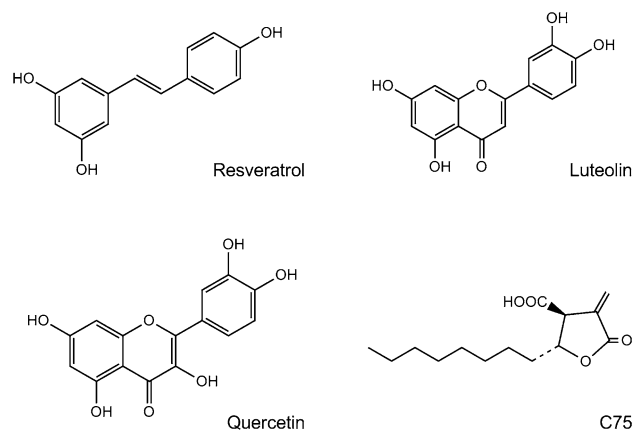


Fig. 1 Structures of compounds tested

(3,5,4'-trihydroxystilbene), is a well-known constituent of grapes (*Vitis vinifera*) and wine, the fermented juice. Luteolin (3',4',5,7-tetrahydroxyflavone) is found in celery, parsley, and other foods (Manach et al. 2004; Fig. 1).

Mass isotopomer distribution analysis is a relatively new technique for characterizing fractional distribution of stable isotope labels by gas chromatography/mass spectrometry (GC/MS) in biomolecules using ^{13}C -labeled precursors. The distribution and rearrangement of labels can be used to fingerprint the biochemical reactions which contribute to the synthesis of the molecule under investigation (Metallo et al. 2009). This technique has been used to characterize carbon deposit patterns of intracellular lipids after labeling media glucose, which led to the discovery that glucose carbons are the main source of cholesterol, palmitate, and stearate in cultured hepatoma cells (Lee et al. 1998b). Glucose carbons are also utilized for building nucleic acids, ribose, and nucleotides from glycolytic precursors and pentose cycle metabolites, but little is known about the contribution of specific synthetic reactions to this process in tumors treated with targeted FAS inhibitors or phytochemicals.

In this study we evaluated a set of flavonoids postulated to have anti-FAS activity on proliferation and apoptosis of pancreatic cancer cells in vitro, and on the metabolic profile of glucose use in these cells after treatment.

2 Materials and methods

2.1 Cell culture and proliferation

MIA PaCa-2 (ATCC CRL1420) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were incubated at 37°C, 5% CO_2 and 95% humidity in DMEM with 10% FBS, and passed using 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA) no more than ten times after receipt from the ATCC prior to use in this study. Cell populations were harvested at 75–80% confluence and plated 24 h before treatment. Five thousand cells were seeded in 100 μl of media in each well of 96-well flat-bottomed microplates. The compounds tested were C75 (Phoenix Pharmaceuticals, Belmont, CA), the polyphenols luteolin and quercetin, and the stilbene resveratrol (Sigma-Aldrich Chemical, St. Louis, MO; Fig. 1), all of which were diluted in 0.1% DMSO. Cells were treated with increasing doses between 0.5–100 μM ; these doses have been used previously in experiments in prostate cancer cells (Brusselmans et al. 2005). Cells were treated for 48 h then harvested for measurement of proliferation and metabolic profiling.

Cell viability was assayed in triplicate using an ATP-based cell proliferation assay, CellTiter-Glo (Promega,

Madison, WI). The software program Prism 4.0 (GraphPad Software Inc., San Diego, CA) was used for analyses of proliferation data. Level of proliferation for each compound tested was compared to vehicle (0.1% DMSO) control. The control value was set at 100% and responses for individual compounds were calculated as a percentage of the control. Mean values were calculated from triplicate wells averaged over three experiments and EC_{50}s (the effective concentration that elicits a half-maximal response) were estimated using non-linear regression analysis with a sigmoidal dose response model provided by the software package.

2.2 Stable glucose isotopes

Stable [1,2- $^{13}\text{C}_2$]-D-glucose isotopes were purchased from Isotec, Inc. (Miamisburg, OH) with >99% purity and 99% isotope enrichment for each position. Unlabeled glucose was purchased from Sigma Chemical Company (St. Louis, MO). For isotope incubation and treatment studies, the cells (5,000/flask) were seeded in T175 (Falcon, Franklin Lakes, NJ) tissue culture flasks, and supplied with 50% naturally labeled and 50% [1,2- $^{13}\text{C}_2$]-D-glucose which were dissolved in otherwise glucose- and sodium pyruvate-free DMEM (Gibco Invitrogen, Carlsbad, CA), with 10% FBS. The final glucose concentration was adjusted to 370 mg/100 ml in each culture. Phytochemicals and C75 were included at both 50 and 100 μM .

Cholesterol and fatty acids were extracted by saponification of Trizol (1.0 ml, Invitrogen, Carlsbad, CA) cell extract after removal of the upper glycogen- and RNA-containing supernatant using 30% KOH and 100% ethanol (300 μl each) for 2 h. Sterol extraction was performed using 5 ml petroleum ether (EMD, Gibbstown, NJ) with repeated shaking for 20 s three times (Crick and Carroll 1987). The molecular ion of cholesterol was monitored at m/z 386 ion cluster. Fatty acids were extracted after cholesterol extraction by acidification with 6 N hydrochloric acid to below pH 4.0 of the Trizol layer. Fatty acids (palmitate) were monitored at m/z 270. The enrichment of acetyl units in plasma and tissue cholesterol and palmitate in response treatment was determined using the mass isotopomer distribution analysis (MIDA) approach. Cholesterol synthesis is dependent on glucose carbons since they are the primary source of acetyl-CoA, the carbon of which is then incorporated into fatty acids and cholesterol by de novo synthesis. Acetyl-CoA and fractions of new synthesis were calculated from the m_4/m_2 ratio using the formula $m_4/m_2 = (n - 1)/2(p/q)$, where n is the number of acetyl units, p is the ^{13}C labeled precursor acetate fraction and q is the ^{12}C labeled natural acetate fraction ($p + q = 1$) (Lee 1996).

RNA ribose and cellular glycogen were isolated by 2 h acid hydrolysis in 2 N HCL of the cellular RNA chloroform-isopropanol fraction after Trizol-purification of cell

extracts (Invitrogen, Carlsbad, CA). Ribose isolated from RNA and glucose isolated from cellular glycogen (Powell et al. 2006) were derivatized to their aldonitrile acetate form using hydroxyl-amine in pyridine and acetic anhydride as described for plasma glucose above. We monitored the ion cluster around the m/z 256 (carbons 1–5 of ribose, chemical ionization, CI) in order to find the molar enrichment and positional distribution of ^{13}C labeled carbons in total cellular messenger, ribosomal and transfer RNA-derived ribose (Lee et al. 1998a).

Lactate from the cell culture media was extracted by ethyl acetate after acidification with HCl. Lactate was derivatized to its propylamide-heptafluorobutyric form and the m/z 328 (carbons 1–3 of lactate, chemical ionization, CI) was monitored for the detection of $m1$ (lactate with a ^{13}C in one position) and $m2$ (double-labeled lactate) for the estimation of pentose cycle activity versus anaerobic glycolysis (Lee et al. 1998a). Glutamate was separated from the media using ion-exchange chromatography (Lee 1996). Glutamate was converted to its *n*-trifluoroacetyl-*n*-butyl derivative and the ion clusters m/z 198 (carbons 2–5 of glutamate, electron impact ionization, EI) and m/z 152 (carbons 2–4 of glutamate, electron impact ionization, EI) were monitored. Isotopomeric analysis of C2–C5 and C2–C4 fragments of medium glutamate was performed in order to estimate the relative contributions of pyruvate carboxylase and pyruvate dehydrogenase to the Krebs cycle.

2.3 Gas chromatography/mass spectrometry

Mass spectral data were obtained on the Agilent 5975 Inert XL Mass Selective Detector connected to an HP6890N Network gas chromatograph. The settings are as follows: GC inlet 230°C, transfer line 280°C, MS source 230°C, MS Quad 150°C. An HP-5 capillary column (30 m length, 250 μm diameter, 0.25 μm film thickness) was used for glucose (plasma, urine or glycogen) and RNA ribose analysis. A ZB-1 100% polydimethylsiloxane (Phenomenex, Torrance, CA., USA) column (15 m length, 250 μm diameter, 0.25 μm film thickness) was used for cholesterol analysis with specific temperature programming for sterol recovery around 25 min retention time. ^{13}C positional enrichment is presented as ^{13}C M1, M2 ... Mn, where M represents mass shift in Daltons (D) with an integer indicating the number of ^{13}C carbons replacing ^{12}C in metabolites.

2.4 Data analysis and statistical methods

Mass spectral analyses were carried out by consecutive and independent automatic injections of 1 μl sample by the automatic sampler; analyses were accepted only if the standard sample deviation was less than 1% of the

normalized peak intensity among repeated injections. Data download was performed by three consecutive manual peak integrations using modified (background subtracted) spectra under the overlapping isotopomer peaks of the total ion chromatogram (TIC) window displayed by the Chemstation (Agilent, Palo Alto, CA) software.

Statistical analyses were performed using the parametric unpaired heteroscedastic, two-tailed independent sample “*t*” test as indicated with 95% confidence intervals, and $P < 0.05$ was considered to indicate significant differences in glucose carbon metabolism in control or treated cells.

3 Results

3.1 Proliferation

The ability of the synthesized FAS inhibitor C75 was tested in relation to a panel of phytochemicals previously shown to have FAS-inhibitory activity for their ability to inhibit proliferation of MIA PaCa-2 pancreatic cancer cells. Treatment of MIA PaCa-2 cells with C75 and selected phytochemicals resulted in a dose-dependent decrease in proliferation (Fig. 2). C75 decreased the proliferation rate throughout the dose range evaluated; the IC_{50} was calculated to be 65 μM (Table 1). Surprisingly, luteolin treatment resulted in a similar dose-dependent decrease in proliferation, with a comparable IC_{50} of 75 μM . The other phytochemicals tested, including resveratrol and quercetin had lesser activity in inhibiting proliferation.

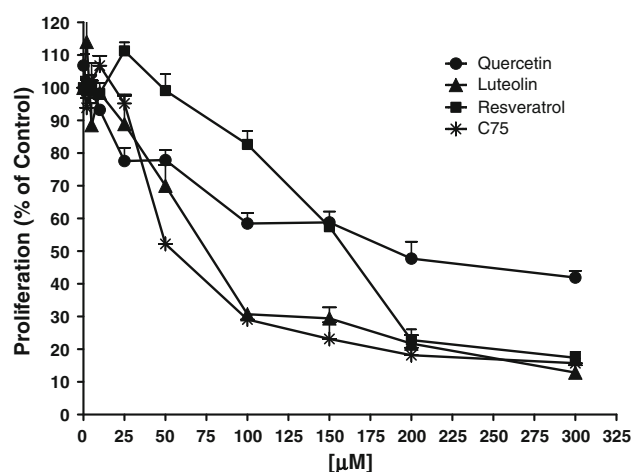


Fig. 2 Proliferation of MIA PaCa-2 cells treated with test compounds. MIA PaCa-2 cells were incubated with quercetin, luteolin, resveratrol or C75 at 0–300 μM . Proliferation was measured by an ATP-based cell proliferation assay. Nonlinear regression analysis was performed using a sigmoidal dose–response model. Data points represent the mean + SEM of three experiments with each dose assayed in triplicate wells

Table 1 IC₅₀s for inhibition of proliferation of MIA PaCa-2 cells by selected compounds

Compound	IC ₅₀ (μM)
C75	65
Luteolin	75
Resveratrol	163
Quercetin	178

3.2 Metabolic profile

Stable ¹³C-labeled palmitate fractions in MIA PaCa-2 pancreatic adenocarcinoma cells were 5.49% in control untreated samples, which decreased to 2.29 and 2.47% after 50 or 100 μM C75 treatment, and to 2.21 and 2.73% after 50 or 100 μM luteolin treatment, respectively (data not shown). Palmitate synthesis via fatty acid synthase, also known as fraction of new synthesis (FNS), was 6.12% of the total ¹³C labeled palmitate pool in control MIA PaCa-2 cells, and decreased to 4.79 and 5.01% with C75 or 1.56 and 0.46% with luteolin (50 and 100 μM doses), respectively (Fig. 3). Resveratrol also reduced the newly synthesized palmitate fraction at the 100 μM dose to 4.33% while quercetin actually increased palmitate synthesis via fatty acid synthase over that of control DMSO-treated cells.

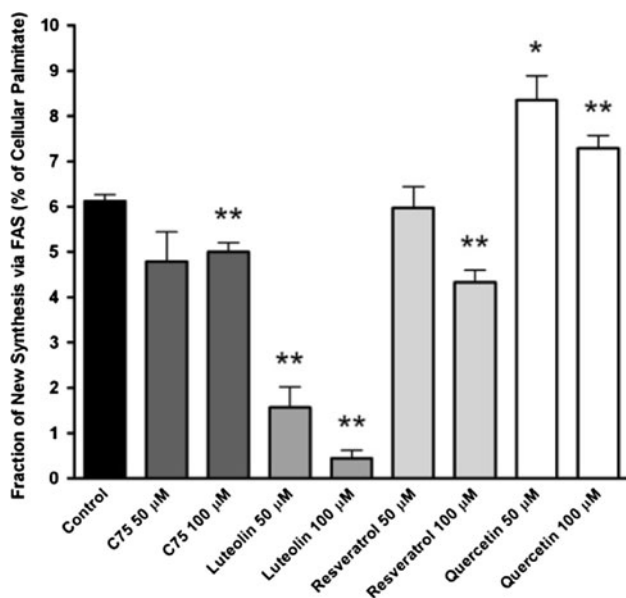


Fig. 3 Newly synthesized lipid (palmitate) fraction via FAS in MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. The newly synthesized fraction of palmitate, which is the only product of the FAS reaction, from glucose, showed a significant decrease at all doses of C75 and luteolin, while luteolin showed a more efficacious inhibition of FAS ($x + SD$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

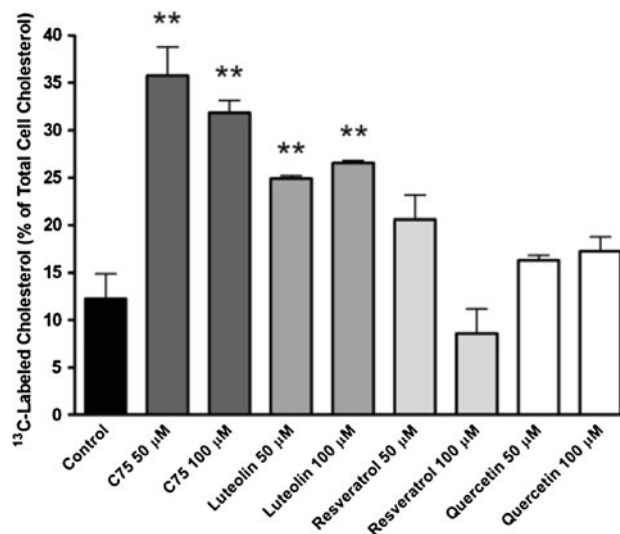


Fig. 4 ¹³C-labeled cholesterol fraction in MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. The ¹³C-labeled fraction of cholesterol from glucose showed a significant increase at all doses of C75 and luteolin. Cholesterol synthesis indicates the alternate route of glucose-derived acetyl-CoA use when FAS is inhibited and therefore is in inversion to that of palmitate labeling and synthesis; thus it can be used as a marker of drug efficacy with in FAS inhibitor treatment ($x + SD$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

While C75 and luteolin effectively inhibited fatty acid synthesis and glucose-derived acetyl-CoA flux via FAS, these treatments increased cholesterol production in comparison with control DMSO treated MIA PaCa-2 cells. This indicates the specific inhibition of FAS but not citrate shuttling from mitochondria for the disposal of glucose-derived acetyl-CoA, which is indicated by the significant 192 and 159% ($P < 0.001$), and 103 and 117% ($P < 0.01$) increase in tracer glucose-derived cholesterol after C75 or luteolin treatment (Fig. 4).

All phytochemicals as well as C75 inhibited glucose to ribose ¹³C labeling of cellular RNA in a dose-dependent fashion; labeling of cellular RNA is an important marker of nucleic acid production and the rate at which MIA PaCa-2 cells are proliferating in culture (Fig. 5). The tracer glucose-derived ¹³C labeled fraction (Σm) of ribonucleotide ribose was 34.73% in controls, which was decreased to 20.58 and 8.45% with C75, 16.15 and 6.86% with luteolin, 27.66 and 19.25% with resveratrol, and 30.09 and 25.67% with quercetin, respectively. Oxidative syntheses of nucleic acid ribose (Fig. 6) as well as the indirect production of lactate by decarboxylation of glucose via G6PDH (direct oxidation, Fig. 7) also decreased; luteolin then C75 (in that order) showed the highest efficacy to decrease G6PDH flux. Inhibition of G6PDH flux deprives cells of NADPH, which in MIA PaCa-2 cells are the main reducing equivalent for deoxyribose and de novo fatty acid synthesis, as

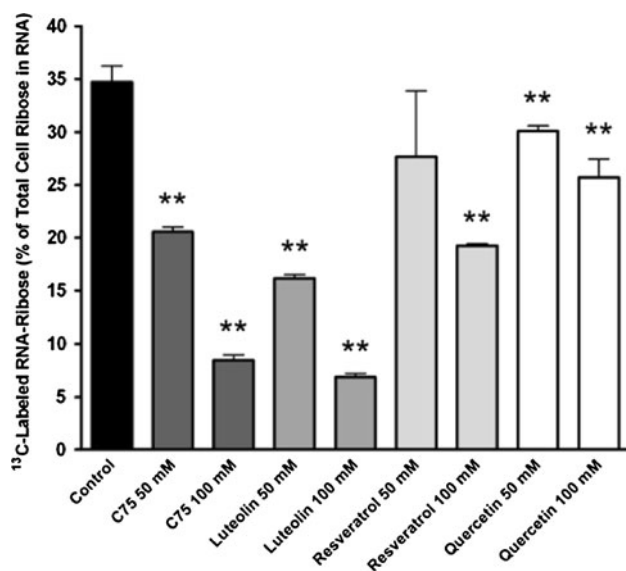


Fig. 5 ^{13}C -labeled RNA-ribose fraction in MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. The ^{13}C -labeled fraction of RNA-ribose from glucose showed a significant decrease at all doses of C75 and phytochemicals. Decreased rates of the nucleotide precursor ribose synthesis indicate decelerated cell proliferation after all treatments ($x + \text{SD}$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

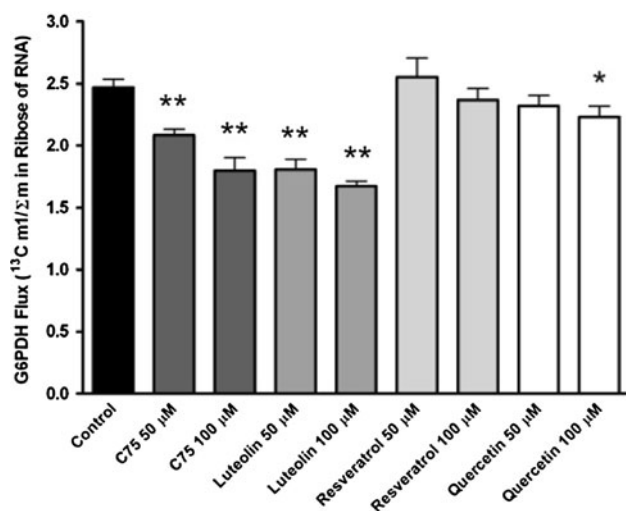


Fig. 6 Glucose-6-P dehydrogenase flux measured by the ^{13}C m1/ Σ m in RNA-ribose of MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. RNA ribose labeled on a single ^{13}C position indicates carbon loss from glucose via direct oxidation and therefore decreased flux via G6PDH and NADPH synthesis in the oxidative branch of the pentose cycle after C75 and more so after luteolin treatment ($x + \text{SD}$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

well as new nuclear and plasma membrane production. Only resveratrol and quercetin at 100 μM inhibited tracer glucose-derived glycogen labeling (Σ m) and turnover by 34.8 and 23.8%, respectively (Fig. 8).

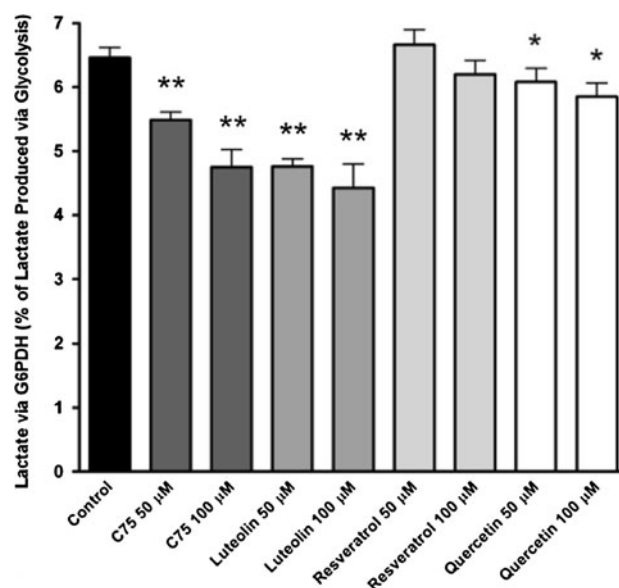


Fig. 7 Lactate (media) ^{13}C -labeling via the oxidative branch of the pentose cycle and G6PDH (^{13}C m1) as percent of lactate produced via glycolysis (^{13}C m2) by MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. Decreased lactate labeled on a single ^{13}C position indicates carbon loss from glucose via direct oxidation and therefore decreased flux via G6PDH and NADPH synthesis in the oxidative branch of the pentose cycle after C75 and more so after luteolin treatment ($x + \text{SD}$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

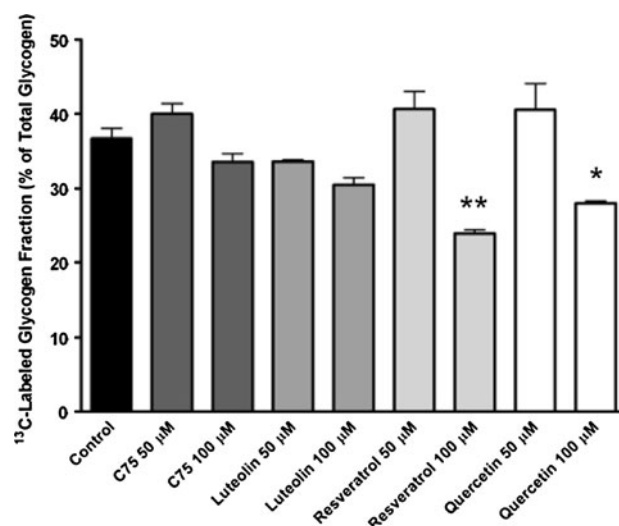


Fig. 8 ^{13}C -labeled glycogen glucose fraction in MIA PaCa-2 pancreatic adenocarcinoma cells in response to increasing doses of C75 and phytochemicals after 48 h of culture. The ^{13}C -labeled fraction of intracellular glycogen glucose showed a significant decrease at 100 μM resveratrol and quercetin treatments. Decreased rates of glycogen turnover have been shown to inhibit cell cycle progression in tumor cells ($x + \text{SD}$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

Basal metabolism of the $[1,2-^{13}\text{C}_2]$ -D-glucose tracer resulted in an average of 24 excess $^{13}\text{CO}_2$ molecules for each 1,000 CO_2 molecule in the culture media, which was

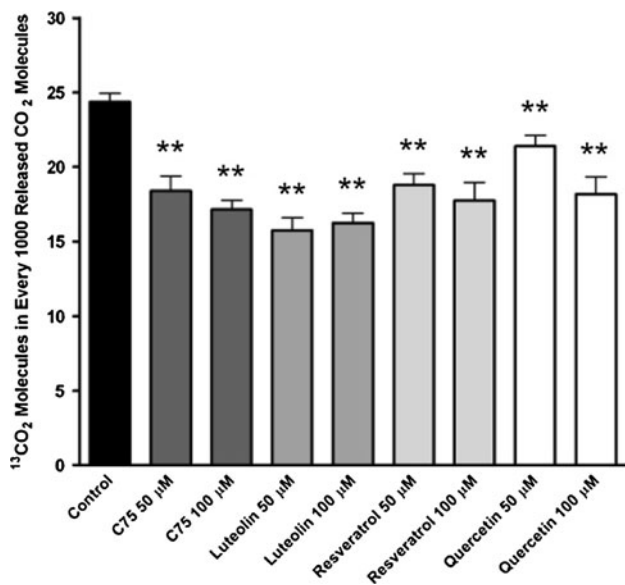


Fig. 9 $^{13}\text{CO}_2$ molecules in every 1,000 released CO_2 molecules in MIA PaCa-2 pancreatic adenocarcinoma cell cultures in response to increasing doses of C75 and phytochemicals after 48 h of treatment. Decreased $^{13}\text{CO}_2$ delta in released CO_2 indicates decreased TCA cycle flux and complete glucose oxidation in mitochondria of MIA PaCa-2 cells after treatment with C75 and all phytochemicals ($x + \text{SD}$; $n = 3$; $P < 0.05^*$, $P < 0.01^{**}$)

decreased by 29 and 33% ($P < 0.01$) with 100 μM C75 and luteolin treatments, respectively (Fig. 9).

Cholesterol's ^{13}C labeling (the sum of all isotopomers up to $M + 6$ as percent of total cholesterol) is relatively high in this study (Fig. 4). Interestingly, the cholesterol content, assayed as the integrated peak area of all ^{13}C labeled and unlabeled cholesterol species in 1 million MIA PaCa-2 cells, show readily decreased concentrations with increased labeling (Table 2). Therefore, the relatively high ^{13}C labeling of cholesterol from glucose with decreased

Table 2 Cholesterol content in MIA PaCa-2 cells with treatment

Treatment	Integrated peak for cholesterol area/million cells	SD
DMSO	2,922	1,449
C75		
50 μM	1,059	38
100 μM	854	53
Luteolin		
50 μM	1,268	225
100 μM	1,483	36
Resveratrol		
50 μM	2,168	904
100 μM	1,470	99
Quercetin		
50 μM	1,892	204
100 μM	1,813	402

cellular concentrations (labeled and unlabeled) after C75 and luteolin treatments clearly indicates high rate of sterol turnover with decreased membrane lipid and cholesterol accumulation in cultures. This is consistent with decreased cell proliferation (membrane expansion) but increased sterol synthesis for other ring modifications after phytochemical treatment. The relatively efficacious lovastatin and other HMG-CoA reductase inhibitor treatments in pancreatic cancer may depend on and here are further validated based on the apparent high rates of cholesterol turnover in MIA PaCa-2 cells (Hussein and Mo 2009).

4 Discussion

Stable ^{13}C substrate-based metabolic profiling linked with mass distribution analysis (MIDA) is the most effective method of characterizing metabolic phenotypes of transformed cells and their response to growth modifying compounds. Specifically labeled isotopic tracers enable the precise characterization of unique reactions within a complex hierarchy of metabolic reactions. The tracer used in this study, $[1,2-^{13}\text{C}_2]\text{-D-glucose}$, provides the most precise measure of substrate to product relationships among tracer substrates for studies of central carbon metabolism that include glycolysis, pentose and TCA cycles, and fatty acid synthesis (Metallo et al. 2009). Specific applications of the use of $[1,2-^{13}\text{C}_2]\text{-D-glucose}$ in animals (Boros et al. 2005) and cell cultures (Yoo et al. 2008) have demonstrated precision with significant translational potential for future metabolic flux analysis (MFA) studies for clinical applications.

Our study reveals a uniform decrease in glucose utilization and deceleration of pancreatic adenocarcinoma cell proliferation via inhibiting pentose (Fig. 5) and TCA cycle substrate fluxes (Fig. 9). Our study also shows, however, the underlying mechanisms by which each of the phytochemicals as well as C75 achieve limited glucose flow via the pentose and TCA cycles are different. C75, as expected, as well as luteolin are very effective in inhibiting de novo fatty acid palmitate ^{13}C labeling from tracer glucose (Fig. 3). This shared mechanism restricts both nuclear and cellular membrane synthesis and thus cell proliferation. Quercetin and resveratrol, on the other hand, are more effective in inhibiting glycogen synthesis and turnover, which has been shown to decrease pancreatic tumor cell proliferation, and induce cycle arrest and apoptosis using the targeted glycogen phosphorylase inhibitor CP-320626 (Lee et al. 2004).

One important finding of our study is that the naturally appearing phytochemical luteolin is more efficacious in inhibiting fatty acid synthase flux than is the targeted pharmaceutical C75 (Fig. 3). ^{13}C labeling from glucose to

palmitate involves several mechanisms, namely FAS flux and heavy carbon exchanges driven by peroxisomal beta oxidation (chain shortening), acetyl-CoA synthesis and chain elongation among the saturated short, medium, and long chain fatty acids of the mammalian cell. Based on our study it is evident that luteolin is more potent in inhibiting fatty acid synthase flux and the ^{13}C labeling of cellular palmitate from glucose-derived acetyl-CoA via FAS-specific reactions. It is very important to note that C75 and luteolin both increase cholesterol synthesis (Fig. 4) from glucose-derived acetyl-CoA as the alternate route of acetate utilization when flux through FAS is blocked. The biological importance of this finding relates to the fact that cholesterol acts as a very effective carbon trap in mammalian cells, including tumors, as the sterol ring cannot be oxidized or broken down to yield energy or other products. In other words, glucose carbons sunk into the sterol ring cannot be returned into the intermediary reactions of the metabolic network. Therefore a major portion of acetyl-CoA derived from glucose is lost for membrane fatty acid synthesis or nucleic acid production in tumor cells treated with C75 and luteolin. This tracer study demonstrates that C75 and luteolin induce metabolic phenotypes consistent with acetyl-CoA-disposing cells via sterol synthesis instead of complete oxidation in the already hypoxic TCA cycle of tumor cells. Increased disposal of acetyl-CoA via citrate shuttling towards cholesterol synthesis in response to C75 and luteolin also indicates that flux via FAS has been deprived; therefore increased cholesterol ^{13}C labeling from glucose may be used as a marker of efficacy of FAS inhibition reflecting the alternate use of acetyl-CoA in the cytosol.

The oxidative branch of the pentose cycle is important for the synthesis of NADPH, the reducing equivalent used by FAS, and ribonucleotide reductase (RNR, also known as ribonucleoside diphosphate reductase) for reducing ribonucleosides into deoxyribonucleosides for DNA replication. Inhibiting FAS decreases demands for NADPH with decreased G6PDH flux (Fig. 6), which deprives tumor cells the ability to synthesize ribose, the 5-carbon backbone of RNA and DNA. This effect of fatty acid synthase inhibitors on the pentose cycle on its own is sufficient to provide FAS inhibitors a significant growth limiting potential in rapidly proliferating transformed cells. In vivo systems may respond differently to targeted fatty acid synthase inhibitor agents with less dependence for de novo fatty acid synthesis as do in vitro-grown cells. This is due to the constant delivery of triglycerides and fatty acids via plasma that can be readily transported into spontaneously growing tumors in animals or humans. Nevertheless, the in vitro growth limiting effects of C75 and luteolin due to FAS inhibition may couple with a rate limiting effect on DNA synthesis in in vivo systems or humans that likely increase efficacy of

these compounds. This likely broadens clinical applications with a clear mechanism of inhibiting DNA replication, besides fatty acid production, and its ribose moiety in the human host.

There are two potential limitations of this study. The first is that we report only cellular palmitate ^{13}C labeling and de novo synthesis results, despite the fact that there are other fatty acids involved in membrane synthesis such as stearate and oleate. Since FAS produces no intermediary products but palmitate, this long chain saturated fatty acid species is an ideal candidate to study the strength of pharmacological compounds to inhibit FAS flux. The second limitation is that carbon disposal mechanisms with resveratrol and quercetin are not as clearly identified as with C75 or luteolin. Therefore, further product analyses may be necessary in tumor cells for quercetin and resveratrol in order to find terminal products of glucose carbon utilization in tumor cells exposed to these phytochemicals. Nevertheless, our findings indicate that limiting carbon recycling and rapid substrate mobilization from glycogen is an effective mechanism underlying the effects of quercetin and resveratrol in rapidly dividing MIA PaCa-2 cells. Growth arrest is closely associated with a characteristic decrease in glycogen breakdown and glucose carbon redistribution towards RNA and fatty acids during CP-320626 treatment (Lee et al. 2004) as well as quercetin and resveratrol in our study. The 35–40% tracer glucose-derived and ^{13}C labeled-cholesterol fractions are high, which are due to the relatively fast/rapid new synthesis of membranes, where the majority of cholesterol gets labeled from glucose when fatty acid synthase is inhibited (C75 and luteolin; pharmacological effect).

This study evaluated the effects of dietary-derived factors on the set of glucose metabolic fluxes. The observed shifts in the metabolic profiles represent the summation of regulatory processes at all levels, including receptor activation, subsequent intracellular signaling, regulation of gene transcription and translation, etc. (Go et al. 2005). Indeed, a lengthy list of anti-carcinogenic activities has been ascribed to the phytochemicals quercetin, luteolin, and resveratrol evaluated here. All are effective anti-oxidants and regulate multiple important cellular signaling pathways, including NF- κ B, Akt, MAPK, Wnt, Notch, p53, the androgen and estrogen receptors and more (Lin et al. 2008; Sarkar et al. 2009). An important consideration in exploring the activities of phytochemicals relative to pharmacological compounds is that they characteristically act in a pleiotropic manner on multiple sites simultaneously, which has important implications to not only the efficacy but the safety of these compounds in cancer prevention (Harris and Go 2006).

In conclusion, the flavonoid luteolin surpasses C75's efficacy to inhibit fatty acid palmitate de novo synthesis as

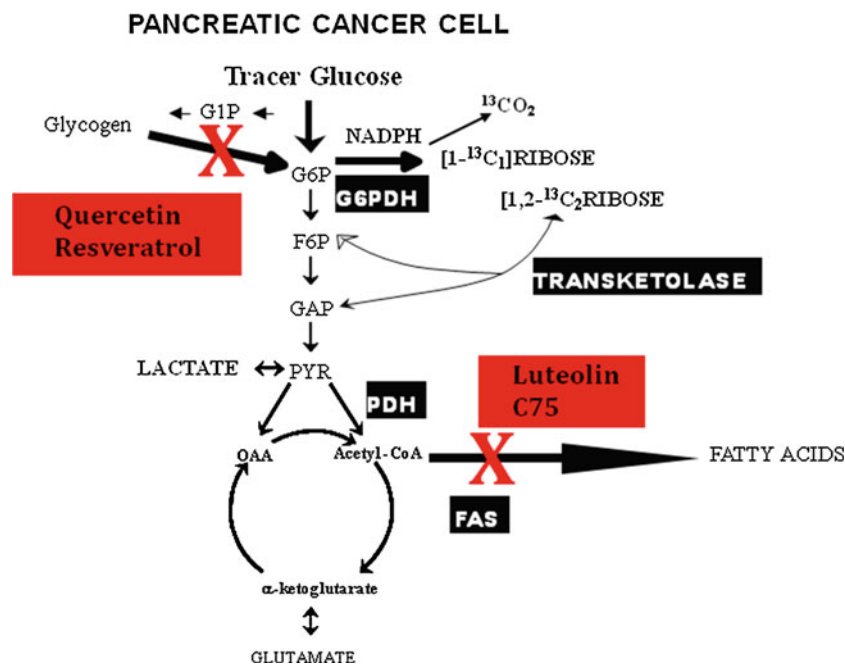


Fig. 10 Summary of metabolic profile changes associated with phytochemical treatment in MIA PaCa-2 pancreatic adenocarcinoma cells. MIA PaCa-2 cells intensely utilize glycogen turnover, non-oxidative ribose synthesis, TCA cycle flux and fatty acid synthase which make these cells sensitive and growth-deprived in the presence of phytochemical or the targeted fatty acid synthase inhibitor pharmaceutical compound C75. (*G6P* glucose-6-phosphate, *G1P* glucose-1-P, *F6P* fructose-6-P, *FAS* fatty acid synthase, *GAP* glyceraldehyde-3-P, *PYR* pyruvate, *OAA* oxaloacetate)

achieve growth arrest is either via inhibiting fatty acid synthase (luteolin) or glycogen turnover (quercetin and resveratrol), as indicated by the X marks. The potency by which luteolin inhibits fatty acid synthase and growth arrest is equal to that of the targeted fatty acid synthase inhibitor pharmaceutical compound C75. (*G6P* glucose-6-phosphate, *G1P* glucose-1-P, *F6P* fructose-6-P, *FAS* fatty acid synthase, *GAP* glyceraldehyde-3-P, *PYR* pyruvate, *OAA* oxaloacetate)

well as nucleotide RNA ribose turnover via the oxidative branch of the pentose cycle. Luteolin is also effective in stringently controlling glucose entry and anaplerosis in the TCA cycle, while it promotes less glucose flux towards cholesterol synthesis than that of C75 (Fig. 10). Quercetin and resveratrol rather inhibit glycogen synthesis and turnover as their underlying mechanism of controlling tumor cell proliferation. Therefore the flavonoid luteolin controls fatty and nucleic acid syntheses as well as energy production with pharmacological strength, which can be explored as a non-toxic natural treatment modality or adjuvant for pancreatic cancer.

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