A novel inhibitor of glucose uptake sensitizes cells to FAS-induced cell death

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Abstract

Evasion of death receptor ligand-induced apoptosis is an important contributor to cancer development and progression. Therefore, molecules that restore sensitivity to death receptor stimuli would be important tools to better understand this biological pathway and potential leads for therapeutic adjuncts. Previously, the small-molecule *N*-[4-chloro-3-(trifluoromethyl)phenyl]-3-oxobutanamide (fasentin) was identified as a chemical sensitizer to the death receptor stimuli FAS and tumor necrosis factor apoptosis-inducing ligand, but its mechanism of action was unknown. Here, we determined that fasentin alters expression of genes associated with nutrient and glucose deprivation. Consistent with this finding, culturing cells in low-glucose medium recapitulated the effects of fasentin and sensitized cells to FAS. Moreover, we showed that fasentin inhibited glucose uptake. Using virtual docking studies with a homology model of the glucose transport protein GLUT1, fasentin interacted with a unique site in the intracellular channel of this protein. Additional chemical studies with other GLUT inhibitors and analogues of fasentin supported a role for partial inhibition of glucose transport as a mechanism to sensitize cells to death receptor stimuli. Thus, fasentin is a novel inhibitor of glucose transport that blocks glucose uptake and highlights a new mechanism to sensitize cells to death ligands. [Mol Cancer Ther 2008;7(11):3546–55]

Introduction

Resistance to stimuli of the death receptor pathway of caspase activation can render malignant cells resistant to chemotherapy, immune surveillance, and anchorage-independent cell death, a process termed anoikis (1, 2). In addition, such resistance would also limit the therapeutic utility of agonistic monoclonal antibodies targeting tumor necrosis factor (TNF) apoptosis-inducing ligand receptors that are now in clinical trial (3). Therefore, small molecules that restore sensitivity of tumor cells to TNF family death receptors would be useful probes to understand this pathway and potentially useful therapeutic adjuncts for the treatment of malignancy.

Diverse mechanisms can create roadblocks to apoptosis triggered by stimuli of the death receptor pathway of caspase activation (4). Documented resistance mechanisms relevant to the death receptor pathway include reduced expression of TNF family death receptors, shedding of soluble death receptors, expression of ligand-binding decoy receptors, reduced expression of caspase-8 and caspase-10, and overexpression of intracellular caspase inhibitors such as the caspase-8 inhibitor c-FLIP (reviewed in ref. 1).

Previously, we used a cell-based high-throughput screen to identify compounds that selectively modulate the extrinsic pathway, sensitizing resistant tumor cells to TNF family death receptors and death ligands (5). From this screen, we identified 5809354 that sensitized cells to death ligands by decreasing expression of FLIP mRNA. This screen also identified the novel FAS-sensitizing chemical compound (*N*-[4-chloro-3-(trifluoromethyl)-phenyl]-3-oxobutanamide; fasentin) that selectively sensitized to death ligands but did not decrease FLIP expression.

The relatively unique 3-oxobutananilide structure of the compound fasentin provided little insight into the possible intracellular targets of this molecule. Therefore, to better understand the targets and pathways affected by this molecule, we analyzed changes in gene expression after treatment with fasentin. These experiments showed that fasentin altered expression of genes associated with nutrient and glucose metabolism. Based on this finding, we explored the effects of fasentin on glucose metabolism and showed that this molecule rapidly inhibited glucose uptake. Subsequent studies supported that the inhibition of glucose uptake of fasentin was functionally important for

Received 6/17/08; revised 8/12/08; accepted 8/27/08.

Grant support: Canadian Institutes of Health Research, Canadian Cancer Society, and Prostate Cancer Research Foundation of Canada, and Ontario Cancer Research Network through funding provided by the Ministry of Research and Innovation in the Province of Ontario.

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi:10.1158/1535-7163.MCT-08-0569

its ability to sensitize cells to death receptor stimuli. Thus, fasentin is a novel inhibitor of glucose transport that blocks glucose uptake and highlights a new mechanism to sensitize cells to death ligands.

Materials and Methods

Reagents

The small-molecule death receptor sensitizer fasentin (Chembridge) was obtained as a powder and stored at room temperature in a glass vial (Chembridge). Stock solutions (100 mmol/L) of fasentin were prepared by diluting fasentin in water with 16% DMSO and were stored at 4°C in polystyrene tubes. Dipyridamole, phloretin, and cytochalasin B were purchased from Sigma-Aldrich. Indinavir was obtained from Merck & Co. CH-11 anti-Fas monoclonal antibody (FAS) to activate the Fas receptor (MBL) was obtained in glycerol and stored at −20°C. 2-[^{3}H]deoxy-D-glucose (20-50 Ci/mmol) was purchased from Perkin-Elmer.

Cell Cultures and Treatments

PPC-1 prostate cancer cells, DU145 prostate cancer cells, and U937 leukemia cells were maintained in RPMI 1640. Primary human peripheral blood mononuclear cells were isolated from fresh peripheral blood samples from healthy volunteers. Mononuclear cells were isolated from the samples by Ficoll density centrifugation. Primary cells were cultured in IMDM. L6 myoblasts stably overexpressing myc-tagged GLUT1 and GLUT4 (ref. 6; L6GLUT1myc and L6GLUT4myc) were cultured in α-MEM. All cells were supplemented with 10% fetal bovine serum (Hyclone), penicillin (500 IU/mL), and streptomycin (50 μg/mL). All cells were cultured at 37°C in a humid atmosphere with 5% CO_2.

The collection and use of human tissue for this study was approved by the local ethics review board (University Health Network).

Real-time Reverse Transcription-PCR

First-strand cDNA was synthesized from 1 μg DNase-treated total cellular RNA from PPC-1 cells using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Real-time reverse transcription-PCR assays were done in triplicate with 10 ng RNA equivalent cDNA, SYBR Green PCR Master Mix (Applied Biosystems), and 400 nmol/L gene-specific primers. Reactions were processed and analyzed on an ABI 7700 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: L-asparagine synthetase (AspSyn; GenBank AY890306; 5-ATGACAGACAGAAGGATTGG-3/5-TACTGAAGCACGAACTGTTG-3), phosphoenolpyruvate carboxykinase-2 (PCK-2; GenBank BC001454; 5-ATGATATTGCTTGGATGAGG-3/5-ACAGTAACACCAGGTGGAAG-3), FLIP-L (GenBank NM-003879; 5-CCTAGGAATCTGCCTGATAATCGA-3/5-TGGGATATACCATGCATACTGAGATG-3), FLIP-S (GenBank U97075; 5-GCAGCAATCCAAAAGAGTCTCA-3/5-TTTTCCAAGAATTTTCAGATCAGGA-3), and GAPDH (GenBank NM-002046; 5-GAAGGTGAAGGTCGGAGTC-3/5-GAAGATGGTGATGGGATTTC-3). Relative mRNA expression was determined using the CT method as described previously (2).

Gene Expression Profiling

Gene expression profiling was done using Affymetrix Human Genome Plus 2.0 with methods recommended by the manufacturer. Genes that were dysregulated at least 2-fold between control and treatment were further validated by real-time quantitative PCR as described above. Identification of gene ontology for genes altered by treatment with fasentin was done using the Database for Annotation, Visualization, and Integrated Discovery. This tool identifies enrichment of gene ontologies comparing the users' gene list against the gene ontology database and scoring for significance (7, 8).

Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay (Promega) according to the manufacturer's protocols as described previously (2). The percent relative cell viability was expressed as [(absorbance of treated cells) / (absorbance of controls)] × 100%.

Apoptosis was measured by Annexin V-FITC and propidium iodide (Biovision Research Products) staining and flow cytometry according to the manufacturer's instructions and as described previously (2).

Assessment of Glucose Uptake

2-Deoxy-D-glucose uptake was done as described previously (6). Briefly, PPC-1, U937, and DU145 cells were grown in RPMI 1640 with 10% (v/v) fetal bovine serum in 24-well plates. Cells were then washed twice with HEPES-buffered saline solution, which contained 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, and 20 mmol/L HEPES (pH 7.4). 2-Deoxy-D-glucose uptake was initiated by addition of 10 μmol/L 2-deoxy-D-glucose and 1 μCi/mL 2-[^{3}H]deoxy-D-glucose in the above HEPES solution at room temperature. 2-Deoxy-D-glucose uptake was terminated by rinsing the cells three times with ice-cold saline solution [0.9% (w/v) NaCl] followed by cell disruption with 0.05 N NaOH. Non-carrier-mediated uptake was determined in parallel wells containing 10 μmol/L cytochalasin B. Protein concentration was measured by the modified Bradford protein assay. Cell-associated radioactivity was determined by scintillation counting. The mean counts/min were expressed as pmol/min/mg protein from determinations done in triplicate. Rates of 2-deoxy-D-glucose were calculated by subtraction of non-carrier-mediated rates from the total rates.

Cell Cycle Analysis

Cell cycle analysis was done as described previously (9). Briefly, cells were harvested, washed with cold PBS, and resuspended in 80% cold ethanol. Cells were then treated with 100 ng/mL DNase-free RNase A (Invitrogen) at 37°C for 30 min, washed with cold PBS, and resuspended in PBS with 50 μg/mL propidium iodide. DNA content was analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

The percentage of cells in each phase of the cell cycle was calculated with ModFit software (Verity Software House).

Inhibitor-GLUT1 *In silico* Interactions

Ligands were built manually and optimized. The previously developed GLUT1 model (PDB entry: 1SUK) based on homology to the crystalline structure of *Escherichia coli* glycerol-3-phosphate antiporter was used for docking studies (10). Docking for each ligand was explored separately using FlexX (11) with default, water-mediated binding variables as implemented in the 7.1 release of the SYBYL package. The most promising of the top 30 (lowest docking score) were considered for comparison of binding sites.

Synthesis of Fasentin Analogues

All solvents and reagents for the chemical syntheses of compounds 1 to 5 were purchased from chemical suppliers and used without further purification. The synthesis of compounds 1 to 5 is detailed below.

Compound 1: 4-chloro-*N*-[4-chloro-3-(trifluoromethyl)-phenyl]benzamide. 4-Chlorobenzoylchloride (0.063 mL, 0.50 mmol) was added dropwise, with stirring, to a solution of 4-chloro-3-(trifluoromethyl)aniline (0.098 g, 0.50 mmol) and triethylamine (0.069 mL, 0.050 mmol) in dichloromethane (3 mL). The reaction mixture was stirred for 4 h at room temperature. The solvent was removed under vacuum. The residual was dissolved in diethyl ether (15 mL) and washed with 10% (v/v) aqueous hydrochloric acid (3 × 10 mL) and distilled water (10 mL). The organic phase was separated, dried with anhydrous magnesium sulfate, and filtered, and the solvent was removed under vacuum. Purification by silica flash chromatography (gradient 5-40% ethyl acetate in hexanes) provided the product as a white powder (77% yield) that was crystallized from dichloromethane/hexanes as colorless crystals.

m.p.: 132-139°C; R_f (60% hexanes, 40% ethyl acetate): 0.79; ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 7.44-7.49 (3H, m), 7.77-7.86 (3H, m), 7.93 (1H, d, J = 2.6 Hz), 8.04 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 119.5 (q, J = 5.6 Hz), 122.7 (q, J = 273.4 Hz), 124.5, 127.7, 128.7, 129.1 (q, J = 31.7 Hz), 129.4, 132.3, 132.6, 136.7, 139.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ(ppm): −63.3; ESI-HRMS calculated 334.0007 for $[C_{14}H_8Cl_2F_3NO + H]^+$, found 334.0024.

Compound 2: 1-Benzyl-3-[4-chloro-3-(trifluoromethyl)-phenyl]urea. 4-Chloro-3-(trifluoromethyl)aniline (0.098 g, 0.50 mmol) was added slowly, with stirring, to a solution of benzyl isocyanate (0.062 mL, 0.50 mmol) in acetonitrile (2 mL). The reaction mixture was stirred for 16 h at room temperature. The solvent was removed under vacuum. Purification by silica flash chromatography (gradient 10-45% ethyl acetate in hexanes) provided the product as a white powder (79% yield).

m.p.: 130-136°C; R_f (70% hexanes, 30% ethyl acetate): 0.38; ^{1}H NMR (400 MHz, DMSO-D_6) δ(ppm): 4.32 (2H, d, J = 5.9 Hz), 6.86 (1H, br t, J = 5.9 Hz), 7.21-7.35 (5H, m), 7.53 (1H, d, J = 8.8 Hz), 7.61 (1H, dd, J = 8.8 Hz, J = 2.4 Hz), 8.09 (1H, d, J = 2.4 Hz), 9.14 (1H, br s); ^{13}C NMR (100 MHz, DMSO-D_6) δ(ppm): 42.8, 116.2 (q, J = 5.5 Hz), 121.5, 122.3, 122.9 (q, J = 272.9 Hz), 126.6 (q, J = 30.5 Hz), 126.7, 127.1, 128.3, 131.8, 140.0, 140.1, 155.0; ^{19}F NMR (282 MHz, DMSO-D_6) δ(ppm): −61.9; ESI-HRMS calculated 329.0663 for $[C_{15}H_{12}ClF_3N_2O + H]^+$, found 329.0677.

Compound 3: 1-Benzyl-3-[4-chloro-3-(trifluoromethyl)-phenyl]thiourea. 4-Chloro-3-(trifluoromethyl)aniline (0.098 g, 0.50 mmol) was added slowly, with stirring, to a solution of benzyl isothiocyanate (0.059 mL, 0.50 mmol) in acetonitrile (2 mL). The reaction mixture was stirred for 16 h at room temperature. The solvent was removed under vacuum. Purification by silica flash chromatography (gradient 10-45% ethyl acetate in hexanes) provided the product as a white powder (99% yield).

m.p.: 153-157°C; R_f (70% hexanes, 30% ethyl acetate): 0.48; ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 4.84 (2H, d, J = 5.2 Hz), 6.22 (1H, br s), 7.30-7.39 (5H, m); 7.50-7.56 (2H, m), 7.85 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 49.8, 117.8, 122.3 (q, J = 273.8 Hz), 124.2 (q, J = 5.4 Hz), 128.0, 128.4, 128.7 (q, J = 31.8 Hz), 129.2, 130.6, 133.3, 135.5, 136.7, 181.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ(ppm): −63.5; ESI-HRMS calculated 345.0434 for $[C_{15}H_{12}ClF_3N_2S + H]^+$ found 345.0436.

Compound 4: *N*-[4-chloro-3-(trifluoromethyl)phenyl]-*N*-methyl-3-oxobutanamide. *N*-Methyl-4-chloro-3-(trifluoromethyl)aniline (0.21 g, 1.0 mmol) was added slowly, with stirring, to a solution of sodium ethoxide (0.078 g, 1.2 mmol) and ethyl acetoacetate (0.15 mL, 1.2 mmol) in xylenes (5 mL). The reaction mixture was stirred for 24 h at 160°C in a pressure-safe sealed vial. The solvent was removed under vacuum. Purification by silica flash chromatography (20% ethyl acetate in hexanes) provided the product as a white powder (47% yield).

m.p.: <25°C; R_f (60% hexanes, 40% ethyl acetate): 0.74; ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): (60% ketone, 40% enol) 1.86 (60% of 3H, s), 2.16 (40% of 3H, s), 3.30 (3H + 60% of 2H, s), 4.70 (40% of 1H, br s), 7.34-7.40 (1H, m), 7.56-7.59 (2H, m), 15.18 (40% of 1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 22.0*, 30.7, 36.7*, 37.6, 50.0, 88.5*, 122.4 (q, J = 273.8 Hz), 126.8, 130.0 (q, J = 31.2 Hz), 132.1, 132.5, 133.3, 142.4, 142.5*, 166.4*, 171.9, 175.7*, 202.1 (* enol tautomer); ^{19}F NMR (376 MHz, $CDCl_3$) δ(ppm): −63.4; ESI-HRMS calculated 293.0430 for $[C_{11}H_{11}ClF_3NO_2 + H]^+$, found 293.0425.

Compound 5: *N*-[4-chloro-3-(trifluoromethyl)phenyl]-3-hydroxybutanamide. Sodium borohydride (0.076 g, 2.0 mmol) was added slowly, with stirring, to a solution of fasentin (1) (0.34 mL, 2.0 mmol) in methanol (6 mL) at 0°C. The reaction mixture was stirred for 30 min at room temperature. The solvent was removed under vacuum and 10% (v/v) aqueous hydrochloric acid was added to the residue until the mixture was neutral. The reaction mixture was extracted with ethyl acetate (3 × 15 mL), dried with anhydrous magnesium sulfate, and filtered, and the solvent was removed under vacuum. Purification by silica flash chromatography (gradient 15-50% ethyl acetate in hexanes) provided the product as a white powder (92% yield).

m.p.: 132-134°C; R_f (60% hexanes, 40% ethyl acetate): 0.12; ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 1.33 (3H, d,

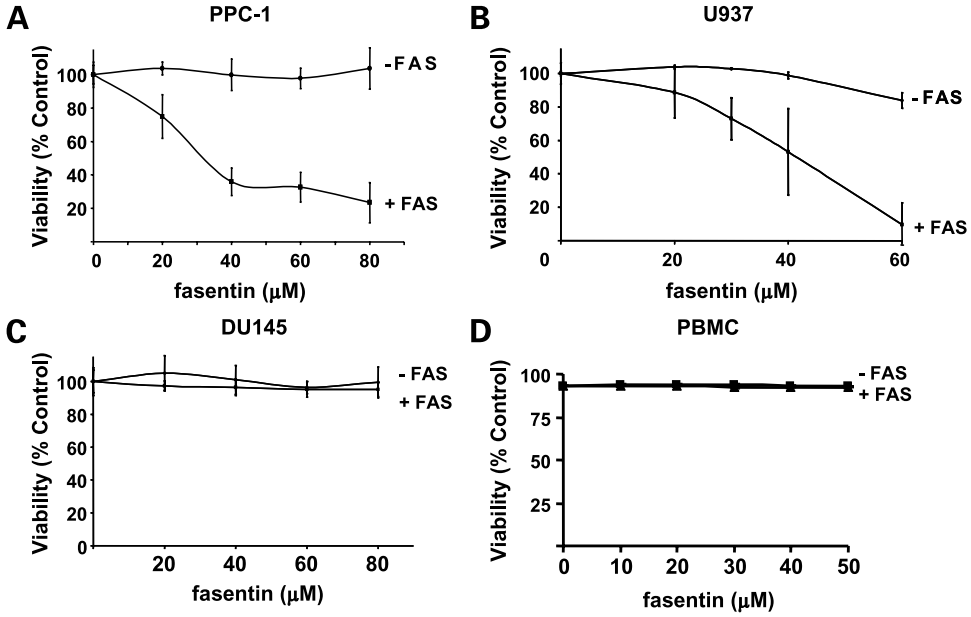


Figure 1. Fasentin sensitizes cells to FAS. PPC-1 prostate (**A**), U937 leukemia (**B**) and DU145 prostate (**C**) cells were treated with increasing concentrations of fasentin with and without CH-11 anti-Fas antibody (FAS; 100 ng/mL). After an overnight incubation, cell viability was measured by the MTS assay. Mean ± SD percent viable cells compared with buffer control (n = 3). **D,** primary human peripheral blood mononuclear cells were treated with increasing concentrations of fasentin with and without FAS (100 ng/mL). After an overnight incubation, cell viability was measured by Annexin V and propidium iodide staining. Mean ± SD percent viable cells compared with buffer control (n = 3).

J = 6.3 Hz), 2.47-2.59 (2H, m), 2.69 (1H, br s), 4.29-4.35 (1H, m), 7.44 (1H, d, J = 8.7 Hz), 7.74 (1H, d, J = 8.7 Hz), 7.82 (1H, s), 8.21 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 23.5, 45.6, 65.2, 119.0 (q, J = 5.6 Hz), 122.8 (q, J = 272.8 Hz) 129.2 (q, J = 31.7 Hz), 124.0, 126.1, 132.2, 136.8, 170.5; ^{19}F NMR (376 MHz, $CDCl_3$) δ(ppm): −63.3; ESI-HRMS calculated 282.0503 for $[C_{11}H_{11}ClF_3NO_2 + H]^+$, found 282.0499.

Results

Fasentin Sensitizes Resistant Cells to FAS and Changes Expression of Genes Associated with Cell Starvation

Via a high-throughput screen for sensitizers to death ligands, we previously identified fasentin that sensitized cells to CH-11 activating anti-Fas antibody (FAS; ref. 5). Figure 1 illustrates that cell viability of PPC-1 prostate cancer cells and U937 leukemia cells was not altered by fasentin alone or by FAS activation alone. However, increasing doses of fasentin sensitized cells to FAS. Reductions in cell viability by MTS assay were confirmed by Annexin V staining (data not shown). In contrast, fasentin did not sensitize DU145 prostate cancer cells to FAS (Fig. 1C). Likewise, fasentin did not sensitize normal resting and phytohemagglutinin-stimulated peripheral blood mononuclear cells to FAS (Fig. 1D; data not shown). Fasentin had a similar effect on sensitizing cells to TNF apoptosis-inducing ligand (ref. 5; data not shown).

To provide insights into the mechanism of action and targets of fasentin, we examined changes in gene expression in PPC-1 cells after treatment with this molecule. PPC-1 cells were treated with fasentin (50 μmol/L) or buffer control for 8 h. After this brief incubation, cells were harvested, and gene expression was measured by microarray analysis (Supplementary Material S1). Included in the list of altered genes were AspSyn (12) and PCK-2 (13) whose up-regulation has been associated previously with nutrient and glucose deprivation. Validating this finding, treatment of PPC-1 cells with fasentin increased expression of these genes by quantitative reverse transcription-PCR at concentrations of the molecule associated with sensitization to FAS (Fig. 2). We also showed up-regulation of AspSyn and PCK-2 after culturing PPC-1 cells in low-glucose medium. Of note, neither fasentin nor low-glucose conditions led to alterations in FLIP mRNA expression, consistent with our previous observations that fasentin sensitized to death receptor stimuli through a mechanism independent of FLIP (Fig. 2B).

Glucose Deprivation Recapitulates Effects of Fasentin

Although the up-regulation of AspSyn and PCK-2 are not likely functionally important for the effects of fasentin as a death receptor sensitizer, these changes in gene expression suggest that fasentin could act through a mechanism associated with glucose deprivation. To test this hypothesis, we cultured cells in low-glucose medium (1 mmol/L) to determine whether glucose deprivation could recapitulate the effects of fasentin. PPC-1 and U937 cells that were sensitized to FAS by fasentin and DU145 cells that were resistant to fasentin were treated with FAS in the presence of decreasing concentrations of glucose. Low-glucose conditions mimicked the effect of fasentin and sensitized PPC-1 and U937, but not DU145, cells to FAS (Fig. 2C). Of note, incubating the cells in the complete absence of glucose induced cell death in all of the treated cell lines (data not shown). Thus, these results suggest that fasentin acts, in part, by promoting partial intracellular glucose deprivation. Furthermore, these results show that partial glucose deprivation sensitizes some but not all cells to FAS.

Fasentin Inhibits Glucose Uptake

Because fasentin appeared to promote intracellular glucose deprivation, we tested whether fasentin altered glucose uptake. Fasentin was added to U937, PPC-1, and

DU145 cells 1 h before measurement of glucose uptake. In U937 and PPC-1 cells, fasentin blocked glucose uptake at concentrations associated with its ability to sensitize cells to FAS (Fig. 2D; data not shown). Inhibition of glucose uptake was also observed in fasentin-resistant DU145 cells. Thus, these results are consistent with the glucose deprivation experiments where culturing cells in low-glucose sensitized PPC-1 and U937 but not DU145 cells to FAS.

To assess the rapidity at which fasentin could block glucose uptake, we added fasentin with 2-[^{3}H]deoxy-D-glucose uptake and measured uptake of the isotope after 5 min. Even with simultaneous addition, fasentin blocked glucose uptake (Fig. 2D). Of note, inhibition of glucose uptake by fasentin was partial and did not exceed 50% at doses between 15 and 80 μmol/L. In contrast, the potent glucose uptake inhibitor cytochalasin B (14) blocked >85% of glucose uptake. Cytochalasin B was also directly cytotoxic to all tested cell lines with a LD_{50} < 10 μmol/L (data not shown), but we cannot exclude mechanisms beyond inhibition of glucose uptake for its cytotoxicity. Thus, fasentin rapidly but partially inhibited glucose uptake.

Fasentin and Glucose Deprivation Arrest Cells in the G_0-G_1 Phase of the Cell Cycle

In addition to altering expression of genes involved in nutrient and glucose metabolism, fasentin also altered expression of genes that regulate cell cycle progression (Supplementary Material S1). Previous studies have shown that glucose deprivation can arrest cells in the G_0-G_1 phase of the cell cycle (15, 16). In addition, some (9, 17–20) but not all studies (21) have reported that arresting cells in the G_0-G_1 phase of the cell cycle can restore sensitivity to death ligands. Therefore, given the changes in gene expression and the reports in the literature, we evaluated the changes

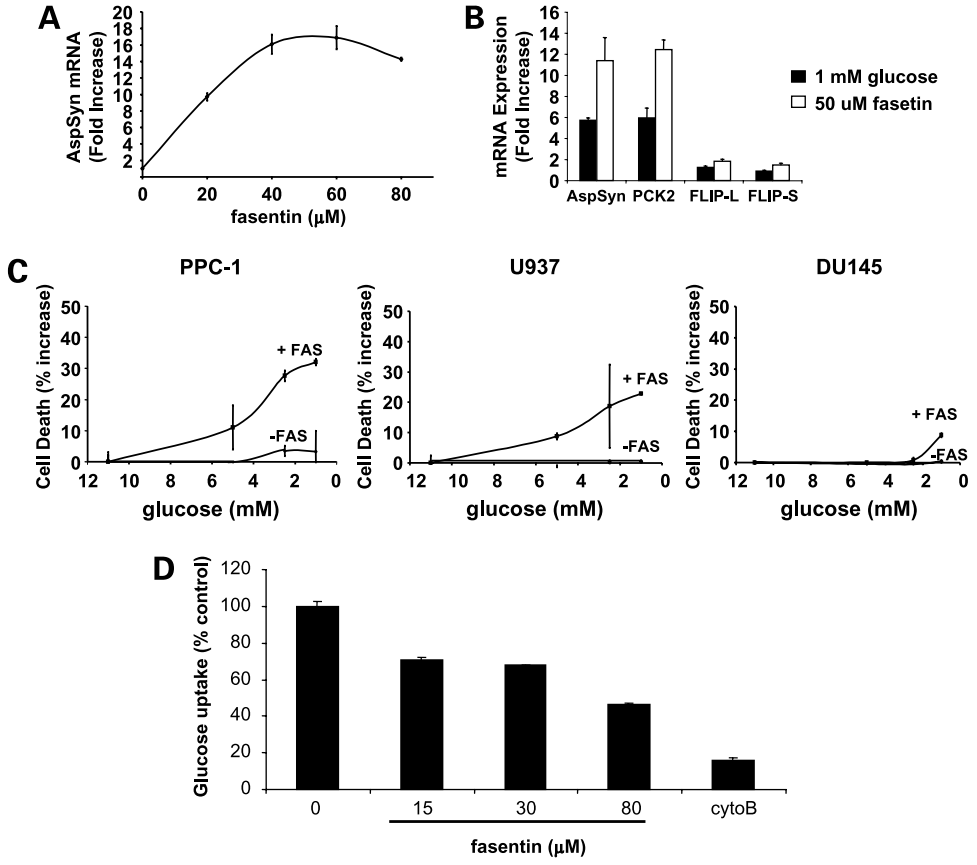


Figure 2. Fasentin induces glucose deprivation, blocks glucose uptake, and arrests cells in the G_0-G_1 phase. **A,** PPC-1 cells were seeded overnight and then treated with increasing concentrations of fasentin for 8 h. After treatment, cells were harvested, total cellular RNA was extracted and mRNA levels of AspSyn or rRNA levels of GAPDH were detected by real-time reverse transcription-PCR. Data were normalized using the $\Delta\Delta C_T$ method and expressed as the mean ± SE fold increase of AspSyn/GAPDH expression relative to untreated controls. **B,** PPC-1 cells were seeded overnight and then treated with fasentin (50 μmol/L) or cultured in low-glucose conditions (1 mmol/L) for 16 h. After treatment, cells were harvested, total cellular RNA was extracted, and RNA levels of AspSyn, PCK-2, FLIP, or GAPDH were detected by real-time reverse transcription-PCR. Data were normalized using the $\Delta\Delta C_T$ method and expressed as the mean ± SE fold increase in expression relative to untreated controls. **C,** PPC-1, DU145, and U937 cells were cultured in medium with decreasing concentrations of glucose with and without FAS (100 ng/mL). After an overnight incubation, cell viability was measured by the MTS assay. Mean ± SD percent dead cells compared with buffer control (n = 3). **D,** increasing concentrations of fasentin were added 1 h before (DU145 and U937) or simultaneously with 2-[^{3}H]deoxy-D-glucose (PPC-1). Cells were also treated with 10 μmol/L of the known GLUT inhibitor cytochalasin B. Five minutes after the addition of 2-[^{3}H]deoxy-D-glucose, the uptake of this glucose analogue was measured as described in Materials and Methods. Mean ± SD percent uptake of 2-[^{3}H]deoxy-D-glucose compared with buffer control treated cells (n = 3).

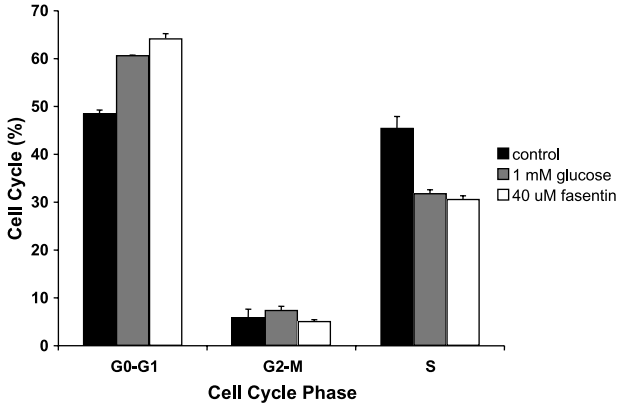


Figure 3. Fasintin arrests cells in the G_0/G_1 phase of the cell cycle. U937 cells were treated with fasentin (40 μmol/L) or cultured in medium with low glucose (1 mmol/L). Sixteen hours after treatment, cells were harvested and the percentage of cells in each phase of the cell cycle was determined by propidium iodide staining and flow cytometry. Mean ± SD percent of cells in each cell cycle phase.

in cell cycle after treatment with fasentin and glucose deprivation to determine whether glucose deprivation was linked to FAS sensitization by promoting cell cycle arrest. Here, U937 cells were treated with increasing concentrations of fasentin or decreasing concentrations of glucose. After 16 h of incubation, the percentage of cells in each phase of the cell cycle was measured by flow cytometry. At concentrations associated with sensitization to FAS and inhibition of glucose uptake, fasentin arrested U937 cells in the G_0-G_1 phase (Fig. 3). Likewise, incubating the cells in low-glucose medium also induced G_0-G_1 arrest. Similar effects on the cell cycle were also observed in PPC-1 cells, but no change in cell cycle was observed in DU145 cells treated with fasentin or glucose deprivation (data not shown). Thus, fasentin and glucose deprivation may sensitize some cells to FAS by promoting cell cycle arrest. In contrast, DU145 cells fail to arrest after fasentin treatment or culture in low glucose medium.

Fasentin Binds a Unique Site on the GLUT1 Transporter in a Virtual Model

Facilitated glucose uptake in cells is regulated by GLUT transporters (22, 23), and rapid inhibition of glucose uptake by fasentin could be due to inhibition of GLUTs. Therefore, we assessed the interaction of fasentin with GLUTs using virtual binding studies. Crystal structures of GLUTs have not been reported previously likely due to the difficulty in crystallizing membrane proteins, but a detailed virtual model of the type I facilitative glucose transport protein GLUT1 has been constructed (10). Therefore, we used this model to assess the partial interaction between fasentin and GLUT1.

Virtual docking using the GLUT1 model (10) developed by homology to the crystalline structure of *E. coli* glycerol-3-phosphate antiporter suggests that fasentin may inhibit glucose uptake by binding the intramembrane channel of the protein. The proposed binding site is unique from binding sites calculated by the same virtual docking program for the known GLUT1 inhibitors dipyridamole, phloretin, and cytochalasin B (Fig. 4A). Thus, these results support fasentin as a unique inhibitor of glucose uptake and suggest that it may act as a GLUT inhibitor.

Fasentin Inhibits Glucose Uptake in Cells Preferentially Expressing GLUT1 and GLUT4

Because fasentin is predicted to interact with the GLUT transporters, we tested its effects in FAS-resistant L6 myoblasts preferentially expressing either myc-tagged GLUT1 (L6GLUT1myc) or GLUT4 (L6GLUT4myc) transporter (6). Fasentin was added to L6GLUT1myc and L6GLUT4myc cells simultaneously with 2-[^{3}H]deoxy-D-glucose and glucose uptake was measured. Fasentin partially inhibited glucose uptake in both cells lines and appeared to inhibit uptake more potently in the L6 cells overexpressing GLUT4 (Fig. 4B). Of note, preincubation of the cells with fasentin for 20 min increased its potency in both L6GLUT1myc and L6GLUT4myc (data not shown). However, the concentration of fasentin required for inhibition of glucose uptake was higher than PPC-1 cell, potentially reflecting the overexpression of the transporters in the transfected L6 cells.

Weak GLUT Inhibitor Dipyridamole Sensitizes PPC-1 Cells to FAS

Given that fasentin might sensitize cells to death ligands by inhibiting GLUTs, we evaluated the effects of other GLUT inhibitors on FAS sensitization. In these experiments, we tested the weak GLUT inhibitor dipyridamole (24), the preferential GLUT4 inhibitor indinavir (25), and the potent GLUT inhibitors phloretin (26) and cytochalasin B (27). Here, PPC-1 cells were treated with increasing concentrations of the GLUT inhibitors with and without FAS, and after an overnight incubation, cell viability was measured (Fig. 5). Likewise, uptake of 2-[^{3}H]deoxy-D-glucose was measured as above. The weak GLUT inhibitor dipyridamole sensitized cells to FAS and, at 100 μmol/L, inhibited glucose uptake by 69 ± 17%. In contrast, the more complete GLUT inhibitors cytochalasin B and phloretin directly induced cell death. Cytochalasin B (10 μmol/L) and phloretin (20 μmol/L) inhibited glucose uptake 85 ± 1% and 84 ± 18%, respectively. In contrast, the selective GLUT4 inhibitor indinavir did not sensitize cells to FAS and did not block glucose uptake. This result is consistent with our finding that PPC-1 cells do not have detectable levels of GLUT4 by immunoblotting. However, GLUT1 was detectable in PPC-1 as well as in U937 and DU145 cells (data not shown). Of note, levels of GLUT1 did not differ among PPC-1, U937, and DU145 cells, indicating that the differential effects of fasentin were not related to variations in GLUT1 expression. Thus, these results suggest that partial inhibition of glucose uptake sensitizes cells to FAS, whereas complete inhibition of glucose uptake is directly cytotoxic.

Evaluation of Glucose Uptake and FAS Sensitization by Analogues of Fasentin

To further define the relationship between fasentin as an inhibitor of glucose uptake and its ability to sensitize cells

to FAS, we used a chemical approach. A series of analogues structurally related to fasentin were synthesized. These analogues were tested for their ability to sensitize cells to FAS and to alter glucose uptake in PPC-1 cells (Table 1). Analogues that maintained an ability to partially inhibit glucose uptake also sensitized cells to FAS and the IC_{50} for sensitization to FAS matched the IC_{50} for inhibition of glucose uptake. Like fasentin, the active analogues only partially inhibit glucose uptake with a maximum inhibition of 70% at a concentration of 80 μmol/L (data not shown). In contrast, analogues that no longer inhibited glucose uptake did not sensitize cells to FAS. Thus, taken together, these results indicate that the ability of the fasentin to inhibit glucose uptake is fundamentally important for its mechanism of action as a sensitizer to death stimuli.

Discussion

Over 80 years ago, it was recognized that malignant cells have increased glucose uptake and abnormal glucose metabolism (28). Known as the ''Warburg effect,'' malignant cells use aerobic glycolysis and preferentially convert pyruvate to lactate even in the presence of oxygen-rich conditions (reviewed in ref. 29). This phenomenon has been attributed to increased expression of factors such as hypoxia-inducible factor-1α and 5′-AMP-activated protein kinase that up-regulate genes involved in glycolysis and down-regulate genes involved in oxidative metabolism of pyruvate (30–32). Recently, the altered glucose metabolism in tumor cells has been linked to exclusive expression of the embryonic pyruvate kinase isoform M2 (33). Therefore, malignant cells should be more susceptible to changes in intracellular glucose and a therapeutic window may be achievable.

In a previous study, we used a high-throughput screen to identify novel sensitizers to stimuli of the death receptor pathway of caspase activation (5). This screen identified the molecule fasentin that sensitized resistant cells to the death ligands FAS and TNF apoptosis-inducing ligand, but its mechanism of action was unknown. Here, we showed that fasentin altered expression of genes associated with glucose deprivation such as AspSyn and PCK-2. Although changes in expression of AspSyn and PCK-2 are not likely to be functionally important for the mechanism of action of fasentin, they provided insight into its potential mechanism. Based on the changes in gene expression, we tested the effects of fasentin on glucose uptake. Fasentin rapidly blocked glucose uptake and this inhibition of glucose uptake appeared functionally important for the ability of fasentin to sensitize cells to FAS.

Inhibition of glucose uptake sensitized PPC-1 and U937 cells to FAS. In contrast, DU145 cells were not sensitized cells to FAS by treatment with fasentin or by culture in low-glucose medium, indicating that altering intracellular glucose levels is not sufficient to sensitize these cells to FAS. Thus, other mechanisms explain the resistance of DU145 cells to FAS. Normal and phytohemagglutinin-stimulated lymphocytes were also not sensitized to FAS by fasentin, suggesting a preferential effect for malignant cells over normal cells.

Glucose uptake into cells is regulated by GLUTs and glucose deprivation could result from inhibition of these transporters. We showed that fasentin binds the GLUT1 transporter using a virtual model. Three lines of evidence support our virtual model and the hypothesis that fasentin sensitizes to cells by inhibiting GLUTs. First, fasentin blocked glucose uptake when added 1 h before or simultaneously with the 2-[^{3}H]deoxy-D-glucose, and this rapid inhibition of glucose uptake is consistent with GLUT inhibition. Second, the weak GLUT inhibitor dipyridamole also partially inhibited glucose uptake and sensitized cells to FAS. Finally, analogues of fasentin that blocked glucose uptake also sensitized PPC-1 cells to FAS, whereas

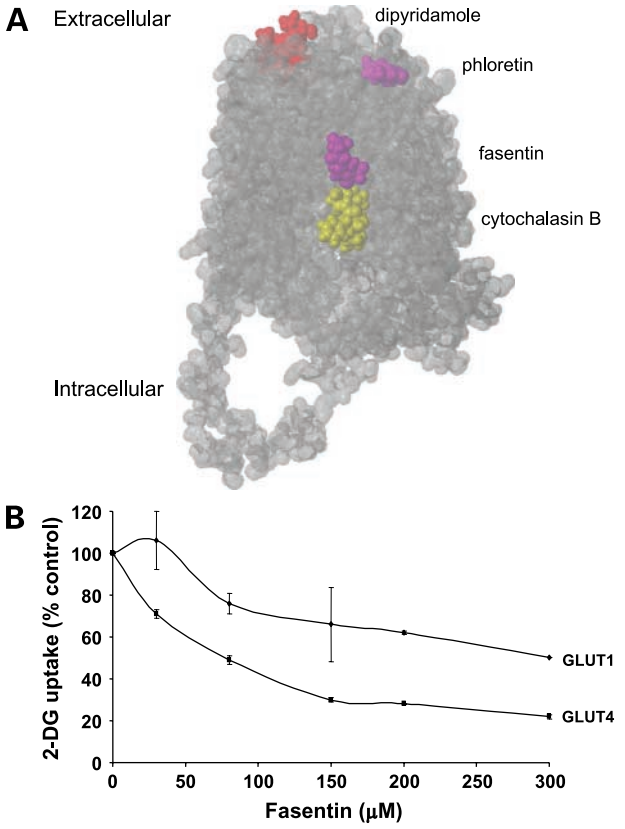


Figure 4. Fasentin binds a unique site on the GLUT1 transporter in a virtual model and inhibits glucose uptake in cells preferentially expressing GLUT1 or GLUT4. **A,** the GLUT1 model based on homology to the crystalline structure of *E. coli* glycerol-3-phosphate antiporter was used for docking studies. Docking for fasentin, dipyridamole, phloretin, and cytochalasin B were explored separately using FlexX with default, water-mediated binding variables. **B,** increasing concentrations of fasentin were added to L6 myoblasts overexpressing GLUT1 or GLUT4 simultaneously with 2-[^{3}H]deoxy-D-glucose. Cells were also treated with 10 μmol/L of the known GLUT inhibitor cytochalasin B. Uptake of the glucose analogue 2-[^{3}H]deoxy-D-glucose was measured as described in Materials and Methods. Mean ± SD percent uptake of 2-[^{3}H]deoxy-D-glucose compared with buffer control treated cells (n = 3).

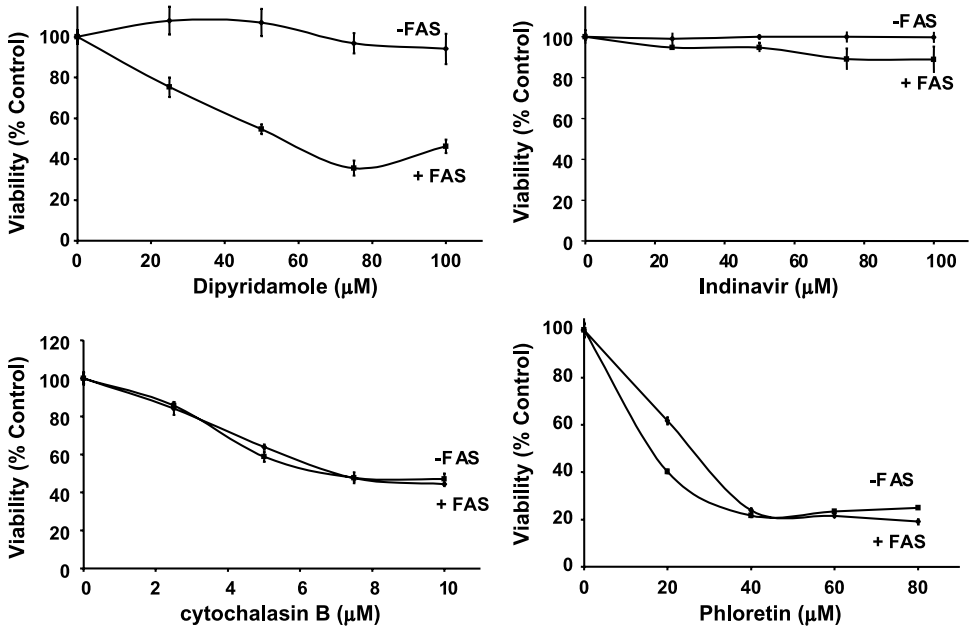
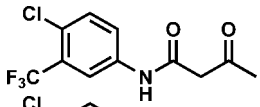
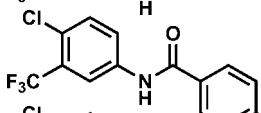
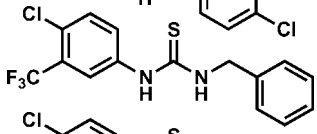
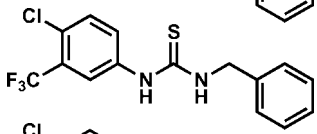
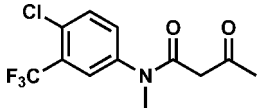


Figure 5. Weak GLUT inhibitor dipyridamole sensitizes PPC-1 cells to FAS. PPC-1 cells were treated with increasing concentrations of dipyridamole, phloretin, cytochalasin B, or indinavir with and without FAS (100 ng/mL). After an overnight incubation, cell viability was measured by the MTS assay. Mean ± SD percent viable cells compared with buffer control (n = 3).

analogues that did not block glucose uptake did not sensitize PPC-1 cells to FAS. Thus, taken together, these results support the GLUT transporter as the target for fasentin. Targeting GLUTs is an attractive therapeutic strategy for the treatment of malignancy, because compared with untransformed cells, malignant cell lines and primary patient samples have increased expression of GLUTs. For example, in patients with breast cancer, GLUT1 overexpression was correlated with advanced tumor grade, increased proliferation, and decreased survival (34). Likewise, the prognostic importance of increased GLUT1 expression has been shown in lung and colorectal cancers (35, 36).

Table 1. Fas sensitization matches inhibition of glucose uptake with fasentin analogues

Identifier	Structure	IC_{50} (-CH-11) (μmol/L)	IC_{50} (+CH-11) (μmol/L)	IC_{50} (glucose uptake) (μmol/L)
Fasentin		>80	20 ± 2	68 ± 6
1		75 ± 7	27 ± 2	65 ± 27
2		72 ± 14	30 ± 2	61 ± 5
3		79 ± 13	37 ± 1	61 ± 17
4		>80	>80	>80
5		>80	>80	>80

NOTE: PPC-1 cells were treated with increasing concentrations of fasentin or compounds 1 to 5 with and without FAS (100 ng/mL). After an overnight incubation, cell viability was measured by the MTS assay. Data are mean IC_{50} ± SD, where the IC_{50} represents the concentration of compound required to reduce cell viability by 50% compared with control treated cells. PPC-1 cells were pretreated with increasing concentrations of fasentin or compounds 1 to 5. Five minutes after treatment, uptake of the glucose analogue 2-[^{3}H]deoxy-D-glucose was measured as in Materials and Methods. Data are mean IC_{50} ± SD, where the IC_{50} represents the concentration of compound required to reduce glucose uptake by 50% compared with control treated cells.

We recognize several limitations of our study. For example, we cannot exclude that fasentin does not inhibit the hexokinase enzyme, as the 2-deoxy-D-glucose uptake assay also measures phosphorylation of glucose. We also concede that fasentin may have binding partners beyond the glucose transport pathway that may also contribute to its ability to sensitize cells to FAS. To further explore the interaction between fasentin and GLUTs, future work will focus on showing a physical interaction between the molecule and the transporter. For example, we would like to confirm the binding of fasentin to GLUT1 transporter by crystallography. However, the crystal structure of GLUT1 has not yet been solved, likely because it is a membrane-bound protein. In addition, future work will explore the specificity of fasentin for the 13 GLUT family members. Using L6 myoblasts preferentially expressing either GLUT1 or GLUT4 (6), we showed that fasentin blocked glucose uptake in both cell lines. Thus, fasentin has inhibitory activity beyond a single GLUT isoform. However, additional studies will be required to elucidate which of the 13 GLUT isoforms are inhibited by fasentin.

Fasentin partially inhibited glucose uptake. In contrast, the strong glucose uptake inhibitors cytochalasin B and phloretin inhibited glucose uptake by >80%. Unlike fasentin, these compounds were directly toxic and did not sensitize cells to FAS. Of note, culturing cells in the complete absence of glucose was also directly toxic. Moreover, complete glucose uptake inhibitors such as cytochalasin B are highly toxic in animal studies (37). Therefore, we speculate that the sensitization to FAS is due to partial inhibition of glucose uptake, whereas complete inhibition directly leads to cell death. If correct, this finding may have implications for developing inhibitors of glucose uptake as therapeutic agents for the treatment of malignancy. Most efforts in drug development focus on developing inhibitors that maximally block their targets with high affinity. Potentially, glucose uptake pathways are targets where submaximal inhibition with a partial antagonist will provide the maximum therapeutic window.

Previous studies have shown that inhibiting glucose metabolism can sensitize cells to death receptor stimuli, but the mechanisms are unclear and at times contradictory (38–40). One previous report showed that culturing DU145 prostate tumor cells in low-glucose medium sensitized them to the death receptor ligand, TNF apoptosis-inducing ligand, by decreasing expression of FLIP protein (39). However, in our sensitized cells, neither fasentin nor low-glucose concentration decreased expression of FLIP. Thus, glucose deprivation is acting through mechanisms other than inhibiting FLIP expression. In some cell lines, sensitivity to death receptor stimuli is increased in cells arrested in the G_1 phase of the cell cycle (9, 17–20). In our study, fasentin and glucose deprivation sensitized PPC-1 and U937 cells to FAS and also induced G_1 cell cycle arrest. In contrast, DU145 cells that were not sensitized to FAS by fasentin and glucose deprivation did not undergo G_1 arrest after either fasentin treatment or glucose deprivation. Thus, the observed G_1 arrest after fasentin treatment and glucose deprivation may be important for conferring sensitivity to FAS. Currently, however, it is uncertain how cell cycle arrest leads to sensitization to death ligands, and future studies will address this issue.

In summary, we have used a chemical biology approach to identify a novel regulator of glucose transport and used it as tool to explore how glucose deprivation sensitizes cells to stimuli of the death receptor pathway of caspase activation. This work may also highlight a new strategy to enhance the clinical activity of death ligands such as TNF apoptosis-inducing ligand.

Disclosure of Potential Conflicts of Interest

A.D. Schimmer is named on a patent involving the therapeutic use of fasentin. No other potential conflicts of interest were disclosed.

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