Orlistat Reduces Proliferation and Enhances Apoptosis in Human Pancreatic Cancer Cells (PANC-1)

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Abstract. Background/aim: Pancreatic cancer is a disease with very poor prognosis, and none of currently available pharmacotherapies have proven to be efficient in this indication. The aim of this study was to analyze the expression of fatty acid synthase (FASN) gene as a potential therapeutic target in proliferating human pancreatic cancer cells (PANC-1), and verify if orlistat, originally developed as an anti-obesity drug, inhibits PANC-1 proliferation. Materials and Methods: The effects of orlistat on gene expression, lipogenesis, proliferation and apoptosis was studied in PANC-1 cell culture. Results: Expression of FASN increased during proliferation of PANC-1. Inhibition of FASN by orlistat resulted in a significant reduction of PANC-1 proliferation and enhanced apoptosis of these cells. Conclusion: This study showed, to our knowledge for the first time, that orlistat exhibits significant antitumor activity against PANC-1 cells. This implies that orlistat analogs with good oral bioavailability may find application in pharmacotherapy of pancreatic cancer.

Pancreatic cancer is a malignancy with particularly unfavorable prognosis. None of currently available chemotherapies have proven to be effective in this indication, and 5-year survival rates for patients with pancreatic ductal adenocarcinoma (PDAC) are no greater than 5% (1). This justifies extensive research on novel, more effective chemotherapeutics that would improve outcomes in this malignancy. Carcinogenesis is associated with substantial changes in cell metabolism of lipids, carbohydrates, nucleic acids and amino acids (1). These changes result in rapid proliferation of cancer cells. More than eight decades ago, Warburg revealed that enhanced anaerobic glycolysis, a phenomenon commonly referred to as the Warburg effect, is a distinctive feature of many human and animal cancer cells (2). During the course of glycolysis, glucose is converted into pyruvate; the latter is either reduced to lactate in cytosol or converted into acetyl-CoA, a substrate for fatty acids synthesis, in mitochondria. As a substrate for phospholipid synthesis, fatty acids are the main component of plasma membranes. The demand for these compounds increases dramatically in quickly-proliferating cancer cells. Fatty acids may also serve as a substrate for protein palmitoylation, a process that is vital for regulation of cancer cell proliferation. Therefore, not surprisingly, previous studies documented up-regulation of enzymes involved in lipid synthesis in many types of cancer, including pancreatic cancer (1, 3).

Fatty acid synthase (FASN) is an enzyme catalyzing synthesis of fatty acids, primarily palmitate. A key role of FASN in carcinogenesis was documented more than two decades ago, when this enzyme was identified as oncogenic antigen-519 (OA-519) in patients with breast cancer (4). FASN is the most widely studied lipogenic enzyme in pancreatic cancer. Its overexpression in pancreatic cancer cells was shown to be associated with poor prognosis (1), as well as with resistance to gemcitabine and radiotherapy (5). This implies that inhibition of FASN might be considered as a therapeutic target in patients with pancreatic cancer. This hypothesis is supported by the fact that treatment with siRNA or FASN inhibitors produced cytotoxic and cytostatic effects in various cancer cells (1). FASN inhibitors include cerulenin, epigallocatechin gallate (a component of green tea extract), luteolin and orlistat, as well as some synthetic agents (1). Antiproliferative activity of two FASN inhibitors, luteolin and C75, was recently documented in pancreatic cancer cell lines (6).

Orlistat (tetrahydrolipstatin, a synthetic derivative of lipstatin produced by Streptomyces toxytrici) is an inhibitor of pancreatic and gastric lipase, reducing absorption of dietary fat. Orlistat was originally developed as an anti-
obesity drug (Xenical®). Its efficacy was confirmed in a study of obese subjects with type 2 diabetes, in whom administration of orlistat combined with lifestyle intervention resulted in greater weight loss and better glycemic control than lifestyle changes alone (7). Orlistat is also an irreversible inhibitor of FASN (8), and several experimental studies demonstrated that through this mechanism, this agent displayed anticancer activity against: prostate tumor xenografts in mice, melanoma cell line B16-F10, breast cancer cell lines, and gastric tumor-bearing mice (9-12). To the best of our knowledge, the effects of orlistat on PANC-1 human pancreatic cancer cells have not been studied thus far. Therefore, the aim of this study was to analyze the anticancer activity of orlistat against PANC-1.

Materials and Methods

Cell culture. PANC-1 Human pancreatic cancer cell line obtained from Sigma-Aldrich (Poznan, Poland) was cultured in low glucose Dulbecco’smodified Eagle’s medium (DMEM) supplemented with heat-inactivated fetal bovine serum (to a final concentration of 10%), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were maintained at 37°C, under a humidified atmosphere with 5% CO2.

Treatment with orlistat. Orlistat (Sigma) was dissolved in dimethyl sulfoxide (DMSO). PANC-1 cells were cultured for 72 h with different concentrations of orlistat (with final concentration of DMSO g 0.25%, v/v) or solely with DMSO (at final concentration of 0.25%, v/v) as a control. Moreover, in order to verify if the study drug really acted as a FASN inhibitor, some PANC-1 cells were pre-incubated with 50 μM palmitic acid for 30 min, and then cultured with palmitate with orlistat for 72 h.

Cell imaging and counting. Cultured cells were photographed with a digital camera coupled with Nikon TMS inverted microscope (Minato, Tokyo, Japan). The cells were then trypsinized and counted with the aid of Penta Square chambers (Medlab Products, Raszyn, Poland).

Analysis of cell viability. After 72-h culture with different concentrations of orlistat (or DMSO as a vehicle control) on 96-well plates, the cell proliferation rate was determined with MTT assay (thiazolyl blue tetrazolium bromide) (Sigma) in line with the manufacturer’s instruction. Absorbance was measured at 570 nm, with BioTek Synergy HT microplate reader (BioTek Instruments Winooski, VT, USA).

Measurement of apoptosis. After 72-h incubation, apoptosis was measured with EnzChek Caspase-3 Assay Kit #1 (Thermo Fisher Scientific, Waltham, MA, USA), in line with the manufacturer’s instruction. Briefly, orlistat-treated and control cells were harvested, washed with phosphate-buffered saline and lysed in Cell Lysis Buffer. After centrifugation, supernatants were transferred to individual microplate wells. Cell Lysis Buffer was used as no-enzyme control to determine background fluorescence and benzzyloxy carbonyl 7-amino-4-methylcoumarin-derived substrate was added to each sample as a substrate. Proteolytic cleavage of the substrate results in the release of fluorescent product. Fluorescence intensity, proportional to the activity of caspase-3 in pretreated cells was measured at 360/460 nm excitation/emission, with BioTek Synergy HT microplate reader.

Determination of mRNA levels of FASN and sterol regulatory element-binding protein 1 (SREBP1) by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from PANC-1 cells using GeneElute Total RNA Purification Kit (Sigma-Aldrich). The RNA concentration was determined based on the absorbance at 260 nm (Beckman DU-640 Spectrophotometer; Beckman-Coulter, Brea, CA, USA). Subsequently, 1 μg of isolated RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), in line with the manufacturer’s instruction. Real-time PCR was conducted with CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using iQSYBR Green Supermix (Bio-Rad) and the following pairs of specific forward (F) and reverse (R) primers: FASN F- CGGAACGGCATCAACCGAGAT, R-CTTGACGCAATGAGTCTC; SREBP1 F-CCGAAAACCTGTTGGACA, R-GCCGGTTGATAGGCAGCTT; β-actin (ACTB) F-TGTGTACACACTGGGACGATA, and R-GGGGTGTGGAAAGCTCCTAAAA. Relative mRNA levels for the analyzed genes were quantified using comparative 2−ΔΔCT method against β-actin as a reference gene.

Determination of lipogenesis rate in PANC-1 cells. Lipogenesis rate was determined based on the incorporation of tritium from 3H2O (2 mCi/ml) into fatty acids during 24-h incubation of PANC-1 cells, as described by Gibbons et al. (13). Lipogenesis was stopped by a 15-min incubation with 1.5 ml 5N KOH, and then, after adding 1.5 ml of 95% ethanol, the samples were acidified with 1.5 ml of sulfuric acid, followed by extraction of lipids with petroleum ether. The petroleum ether layer was washed twice with 5 ml of water. Radioactivity of the lipid fraction was determined with a Beckman LS 6000 IC liquid scintillation counter (Beckman-Coulter).

FASN activity assay. Activity of FASN was measured spectrophotometrically based on the rate of NADPH oxidation (14). Briefly, PANC-1 cells were lysed in a lysis buffer (20 mM Tris-HCl (pH 7.8), 0.2% Triton X-100). The lysates were centrifuged, and protein content in supernatants was determined with Lowry’s method. Supernatants were incubated at 37°C with 0.1 M potassium phosphate (pH 7.0), 0.2 M EDTA, 1 mM dithiothreitol, 0.15 mM NADPH, 25 μM malonyl-CoA and 100 μM acetyl-CoA. FASN activity was determined with Beckman DU-640 Spectrophotometer, based on a decrease in absorption at 340 nm resulting from NADPH oxidation. The results were expressed in nmol/min/mg of protein.

Statistical analysis. The results are presented as the mean±SEM. Statistical significance of intergroup differences was verified with one-way analysis of variance (ANOVA) with Holm-Sidak post-hoc test. The results were considered significant at values of p<0.05.

Results

PANC-1 cells were cultured for 4 days. A gradual increase in both FASN mRNA level and FASN activity was observed during the incubation, up to 200% and 400% that of respective baseline levels on day 4 (Figure 1A and B). The
increase in FASN mRNA level was associated with an increase in mRNA level for SREBP1, the main transcription factor regulating FASN expression (Figure 1C). The rate of fatty acid biosynthesis on day 4 was approximately 50% higher than at baseline (day 1; Figure 1D). Treatment of PANC-1 cells with orlistat for 72 h was reflected by a dose-dependent decrease in the cell number and viability (Figure 2A-C). Viability of cells treated with 50 μM orlistat corresponded to approximately 50% that of untreated cells. Furthermore, treatment with orlistat induced some morphological changes of PANC-1 cells, as shown by a larger proportion of cells with a spherical shape (Figure 2B). Moreover, orlistat stimulated a dose-dependent increase in caspase-3 activity in PANC-1 cultures (Figure 2D). Activity of caspase-3 in cultures treated with 50 μM orlistat was approximately 3-fold higher than in control cells. Finally, treatment with 50 μM orlistat caused inhibition of fatty acid biosynthesis in PANC-1 cells (Figure 2E). In order to verify if the antiproliferative effect of orlistat resulted from depletion of the main FASN product, palmitate, PANC-1 cells were incubated with both orlistat and a surplus of palmitate. As shown in Figure 3, the addition of palmitate to PANC-1 cultures eliminated the antiproliferative effect of orlistat.
Figure 2. Influence of increasing orlistat concentration on PANC-1 cell number (A), morphology (B), viability (C), apoptosis (D) and lipogenesis rate (E) in cells cultured for 72 h. Morphological changes were evaluated under a light microscope at ×100 magnification. Cell counts were determined under a microscope with an aid of a hemocytometer. Cell viability was determined with an MTT assay. Apoptosis rate was measured based on caspase-3 activity. The results originate from at least three independent experiments, data are the mean±SEM. *Significantly different from the control cell culture without orlistat at p<0.01.
Discussion

This study showed that proliferation of PANC-1 was associated with a significant increase in FASN expression. This observation is consistent with the results of previous studies (5,15,16) that also documented overexpression of FASN in pancreatic tumor tissue. The increase in FASN expression seems to result from up-regulation of SREBP1 transcription factor, the main regulator of fatty acid synthesis (17,18). Indeed, overexpression of SREBP1 has already been observed in pancreatic cancer tissue (19). Increased expression of FASN results in enhanced production of fatty acids that are necessary for both synthesis of cell membrane lipids and proliferation of cancer cells; furthermore, overexpression of FASN contributes to palmitoylation of proteins involved in carcinogenesis (1). Based on this evidence, FASN seems to be an attractive target for pancreatic cancer pharmacotherapy. Even more importantly, overexpression of this factor was shown to be associated with resistance of pancreatic cancer cells to gemcitabine and radiotherapy (5).

In this study, we tested orlistat, an agent that has already been authorized for pharmacotherapy of obesity and type 2 diabetes mellitus (20), and its antiproliferative potential has been documented in prostate cancer cells (9, 23, 24), squamous cell carcinoma of the tongue (25), glioblastoma cells (26), ovarian cancer cell line SKOV3 (27), NUGC-3 gastric cancer cells (12), human colorectal carcinoma-bearing mice (28), myeloma cells (29), liposarcoma cells (30), T-cell lymphoma (31), retinoblastoma (32), breast cancer cells (10) and mouse melanoma B16-F10 cells (33). However, it was unclear if these antiproliferative effects resulted from depletion of palmitate, the main product of FASN. Our findings presented here imply that this is indeed the case. Firstly, incubation with orlistat contributed to a decrease in fatty acid synthesis in PANC-1 cells. Secondly, the antiproliferative effect of orlistat was abolished.
by addition of palmitate (a product of FASN) to the pancreatic cancer cell culture (Figure 3). Inhibition of proliferation and induction of apoptosis were therefore likely associated with depletion of fatty acids necessary for the synthesis of cell membrane lipids. Taking into account the potential application of orlistat in pharmacotherapy of pancreatic cancer, it should be remembered that antiproliferative properties of this agent may be modulated by the blood (and perhaps also tissue) concentration of palmitate.

Aside from FASN inhibition, orlistat was also postulated to exert its antiproliferative effect via other mechanisms, such as induction of apoptosis, or reduction of proliferation rate due to direct toxicity and stimulation of intracellular accumulation of reactive oxygen species (34). However, our findings suggest that in the case of pancreatic cancer cells, reduced proliferation is a consequence of orlistat-mediated inhibition of FASN.

Potential application of orlistat as a systemic therapy for pancreatic cancer may be substantially limited due to the low cell membrane permeability of this agent, and its poor water solubility (35), oral bioavailability and metabolic stability (36). However, these limitations can probably be overcome, since several orlistat analogs have been synthesized recently (37, 38). Hopefully, at least one of the novel orlistat analogs will satisfy the criteria for use as a systemic therapy against pancreatic cancer.

In conclusion, this study showed that overexpression of FASN gene resulted in a significant increase in PANC-1 proliferation rate. However, inhibition of FASN by orlistat caused a decrease in proliferation and stimulated apoptosis of PANC-1 cells. These findings imply that orlistat may exert an anticancer effect in human pancreatic cancer cells (PANC-1), similar to that observed in other cancer cell lines. Consequently, orlistat analogs with good oral bioavailability may find application in pharmacotherapy of pancreatic cancer.

Acknowledgements

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References