Lipids
DOI 10.1007/s11745-009-3298-2

ORIGINAL ARTICLE

Inhibition of Fatty Acid Synthase by Orlistat Accelerates Gastric Tumor Cell Apoptosis in Culture and Increases Survival Rates in Gastric Tumor Bearing Mice In Vivo

Shawn Dowling · James Cox · Richard J. Cenedella

Received: 24 October 2008 / Accepted: 16 March 2009

Abstract Orlistat, an anti-obesity drug, is a potent inhibitor of fatty acid synthase (FAS) and tumor cell viability. It can also induce apoptotic cancer cell death. We examined the effects of Orlistat on cultured NUGC-3 gastric cancer cells. We identified that inhibition of FAS via Orlistat exposure results in rapid cellular damage preceded by a direct but short-lived autophagic response. The Orlistat induced damage can be reversed through the addition of lipid containing media in a process that normally leads to cell death. By limiting exogenous lipid availability and inhibiting FAS using Orlistat, we demonstrated both a greater sensitivity and amplified cancer cell death by activation of apoptosis. We have identified "windows of opportunity" at which time apoptosis can be aborted and cells can be reversed from the death pathway. However, when challenged beyond the window of recovery, cell death becomes all but certain as the ability to be rescued decreases considerably. In vivo examination of Orlistat's ability to inhibit gastrointestinal cancer was examined using heterozygous male C57BL/6J APC-Min mice, which spontaneously develop a fatal gastrointestinal cancer. Mice were fed either a high fat (11%) or low fat (1.2%) diet containing no Orlistat or 0.5 mg Orlistat/g of chow. Orlistat treated mice fed the high fat, but not low fat diet, survived 7–10% longer than the untreated controls.

Keywords Thin layer chromatography · Analytical Techniques · Lipid biochemistry · General Area · Lipases · Lipoproteins · Metabolism · Cancer · Physiology · Fatty acids · Specific Lipids · Sterols

S. Dowling · J. Cox · R. J. Cenedella (✉)
Biochemistry, A.T. Still University of the Health Sciences, Kirksville, USA
e-mail: rcenedella@atsu.edu

Introduction

Fatty acid synthase (FAS) functions to form palmitic acid from acetyl and malonyl coenzymeA. It is highly expressed in many tumors that are dependent on de novo synthesis to supply the fatty acid needed for growth and proliferation [1–5]. The capacity of FAS inhibitors to kill cancer cells suggests a new approach to treatment of human cancers [6]. Orlistat is one of these FAS inhibitors.

Orlistat, an over-the-counter anti-obesity drug, decreases absorption of dietary fat by inhibiting gastric and pancreatic lipases through covalent modification of the enzymes [7]. It is also a potent inhibitor of FAS [8]. Using an activity-based proteomics screen for serine hydrolases, Kridel et al. [8] discovered that Orlistat irreversibly inhibits the thioesterase activity of FAS. The capacity of Orlistat to inhibit the proliferation of prostatic cancer cells cultured in serum free media appeared to be due to restricting the availability of fatty acids for growth, since the addition of palmitic acid to the media reversed the inhibition [8]. A decrease in DNA synthesis, arrest of cell progression through the G1/S boundary, and apoptotic cell death are all consequences of inhibiting FAS in cultured cancer cells with Orlistat [6, 9].

The effects of Orlistat on proliferation and viability of tumor cells have typically been examined after 24–72 h [8, 10] and even after 4–5 days [10] of continuous exposure to the drug. This approach assumes that the Orlistat has a linear-accumulative effect on tumor cells. Possible immediate or very rapid effects of Orlistat on tumor cells have not been reported. We searched for such effects and

Published online: 21 April 2009

observed that as little as one-half hour exposure of NUGC-3 cells to 100–500 μM Orlistat induced transient autophagy. In addition, we observed that 4 h exposure followed by removal of the drug reduced viability when examined at hour 48. The presence of Orlistat for the first 8 h of culture resulted in near total loss of viability when measured 40 h later. Acute-irreversible damage appeared to occur at the interface between 4 and 8 contact hours with Orlistat. We probed the mechanism of reduced viability by examining NUGC-3 cells for the emergence of autophagy and apoptosis following short-term exposure to the drug. Rapid onset of apoptosis was attributed to nutritional stress, in which altering the availability of nutrients places a stress on the cell to acquire alternative sources of nutrients to meet the cells biogenesis needs. In this case, nutritional stress was caused by the deprivation of fatty acids since the apoptosis could be arrested by addition of whole serum to the culture media.

Although Kridel et al. [8] demonstrated that intraperitoneally injected Orlistat suppressed the growth of implanted tumor cells, use of Orlistat to treat systemic tumors is limited by the fact that it is essentially unabsorbed following oral dosage [11]. However, since orally administered Orlistat would directly contact the gastrointestinal mucosa, the drug might be of value in treating gastrointestinal tumors exposed to the intestinal lumen. We tested this possibility by examining the effect of orally given Orlistat on the survival of C57BL/6J APC-Min mice (Min). The Min mice provide an excellent in vivo model for human gastrointestinal cancer, since the adenomas that develop in these animals result from inactivation of the same tumor suppressor gene associated with most human colon cancers [12]. The tumors develop due to a mutation in the murine AP gene, a homolog of the human APC gene (JAX MICE literature, The Jackson Laboratory). However, Min mice develop a heavy tumor load in the small rather than large intestines which results in death at several months of age [13–15].

Materials and Methods

Cell Culture and Reagents

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. NUGC-3 gastric cancer cells (human stomach) were obtained from the Health Science Research Resource Bank (Osaka, Japan). Bovine lens epithelial cells (local source) were used as a normal cell control. Cells were seeded at 30,000–40,000 per well in 96 well dishes and grown at 37°C and 5% carbon dioxide in RPMI-1640 media containing 10% fetal calf serum (FCS) plus 0.25% antibiotic/antimycotic containing mix. This is called whole media (WM). Experiments were started 1–2 days later with cell layers 70–90% confluent. Orlistat from 5 to 120 mg Zenical™ capsules (obtained from a local pharmacy) was extracted by homogenization in 3–5 ml aliquots of 1:1 (v/v) chloroform:methanol and transferred to a weighed test tube. After evaporation of the solvent under nitrogen and further drying overnight under vacuum, the recovered Orlistat was dissolved in ethanol at 50 mg/ml. Aliquots were added to WM or serum deficient media (DM) containing 0.1% FCS to yield 500 μM. Serial dilutions provided media containing 200, 100, 50, and 25 μM Orlistat. The capacity of Orlistat to decrease cell viability was also examined in non-cancerous (normal) bovine lens epithelial cells. Culture conditions were as we described before [16].

Cell Viability

Cell layers in groups of 5 or 6 wells were incubated for 4–48 h at 37°C with 200 μl of WM or DM containing 0–500 μM Orlistat. Cell viability was assessed by the MTT (thiazolyl blue tetrazolium bromide) assay essentially as described by Carmichael et al. [17]. Test media was replaced with 200 μl of WM, 25 μl of MTT solution (5 mg/ml water) was added, and the samples incubated for 4 h at 37°C. The MTT solution was replaced with 100 μl of DMSO and the optical density at 570 nm measured 1 h later. Absorbance for each group was expressed as the mean ± SEM. Statistical significance of differences ($P \leq 0.05$) was assessed by one-way ANOVA.

Measurement of Fatty Acid and Synthesis

Near confluent layers of NUGC-3 cells were washed twice in serum free media, after which 200 μl of plain RPMI-1640 media was added followed by 1 μl of ^{14}C-acetate (55 mCi/mmol, ARC Inc, St. Louis, MO). Serum free media was used to avoid dilution of the labeled acetate with competing sources of unlabeled acetate. Following 4-hour incubation in the absence or presence of varying concentrations of Orlistat, cell layers were washed twice with PBS. The cell layer in each well was dissolved in 200 μl of 1 N NaOH in 50% ethanol containing 100 μg of triolein and 100 μg of cholesterol added as carrier. Each well was washed with 4 additional 200 μl-aliquots of the 1 N NaOH and pooled into screw capped test tubes with the first 200 μl. Samples were saponified for 2 h at 100°C, diluted with 1 ml of water, acidified and the non-saponifiable plus saponifiable lipids extracted with 2–4 ml aliquots of hexane. The hexane extracts were washed with an equal volume of water to remove any water-soluble radiolabel. The hexane extracts were evaporated and the lipids separated by thin layer chromatography in silica gel

using a solvent of hexane:diethyl ether:glacial acetic acid (73:25:2: v/v/v). The sterol (cholesterol) band and the fatty acid band were recognized by exposure to iodine vapor and marked. After loss of the iodine by sublimation, the cholesterol and fatty acid bands were recovered and the radiolabel measured by scintillation counting.

We accounted for variation in incorporation from well to well in any given group by expressing the level of fatty acid synthesis as a ratio of incorporation of ^{14}C into fatty acid to cholesterol. Cholesterol controls were run to determine Orlistat's effect on total sterol synthesis. Significant differences were not seen in the disintegrations per minute (dpm) of ^{14}C-acetate incorporated into cholesterol between control and Orlistat treated cell layers. Decreases in the fatty acid:cholesterol ratio reflects inhibition of fatty acid synthesis, since Orlistat has no known effects on cholesterol biosynthesis.

Western Blotting: Changes in the Relative Mass of Fatty Acid Synthase (FAS)

NUGC-3 cells were grown in RPMI-1640 on 35 mm plastic dishes to assure adequate protein. Cell layers exposed or unexposed to Orlistat under varying condition were washed twice with PBS and dissolved in 0.50 ml of lysis buffer [18]. Samples were assayed for protein (aliquots containing 50 μg protein were separated on 8% PAGE gels 94% stacking gel), transferred to membranes which were then probed with 0.8 μg/ml rabbit-polyclonal, antihuman FAS-antiserum (Santa Cruz Biotechnology) and HRP-conjugated goat-antirabbit IgG secondary antibody (1/60,000 dilution). Immunoreactive protein was detected by enhanced chemiluminescences and relative concentrations estimated by densitometric scanning of the exposed X-ray film as done before [18].

Detection of Autophagy and Apoptosis

Autophagic vacuoles were labeled with monodansylcadaverine (MDC) using a modified protocol as described by Biederbick et al. [19]. Briefly, cells were grown on coverslips, incubated at 37°C in WM, and DM ± 500 μM Orlistat for varying time intervals. Media was removed; cells were washed once with PBS, and replaced with PBS supplemented with 0.1 mM MDC. Cells were allowed to incubate for 10 min at 37°C. Following incubation, cells were washed three times with PBS, fixed using 4% paraformaldehyde, and mounted using Fluoromount. Cells were examined using a NIKON Eclipse 80i upright fluorescent microscope equipped with a V2-A filter system (excitation 340 nm, barrier 514 nm). Images were captured using a NIKON DS-Qi1 digital camera and were processed using Image J.

Autophagic vacuole formation was measured using methods described by Munafo and Colombo [20]. Briefly, cells were grown in 60 mm dishes and treated using varying concentrations of WM and DM ± Orlistat. To identify autophagy activity and duration, MDC incorporation was measured after 0.5, 1, 2, 4, 6, 8, and 10-hour Orlistat exposure. After varying times the media was removed and PBS was added with the addition of 0.1 mM of MDC and incubated at 37°C for 10 min. Following incubation the cells were washed three times with PBS and collected in 10 mM Tris–HCl, pH 8 with 0.1% Triton X-100. Intracellular MDC incorporation was measured using a BioTek FLx 800 Microplate Fluorescent Reader equipped with an excitation filter of 365/20 nm and an emissions filter of 528/20 nm. The number of cells per well were normalized by the addition of ethidium bromide to a final concentration of 0.1 μM to each well followed by the measuring of DNA fluorescence (excitation filter 528/20 nm and emission filter of 590/20 nm). The MDC measured was expressed as the percent of autophagic activity relative to whole media control.

The mechanism of cell death (apoptosis) was determined through the use of fluorochrome inhibitor of caspases (FLICA) (Immunochemistry Technologies, LCC). Activated caspase is an enzyme found active only in cells undergoing apoptosis. FLICA irreversibly binds to caspases (caspase-1, -3, -4, -5, -6, -7, -8, -9) allowing apoptosis to be measured [21, 22]. Caspase activity was visually examined using the suggested manufactures protocol for labeling caspases with FLICA. Briefly, cells were grown on coverslips and treated with various concentrations of Orlistat substituted in DM. Cells were then treated with FLICA solution in a 1:30 ratio (10 μl FLICA:290 μl media) and incubated for 1 h. Next, media was removed and replaced with fresh media (300 μl) containing 1.5 μl of 200 μg/ml stock Hoechst stain solution (Immunochemistry). After 5 min cells were rinsed with wash buffer (Immunochemistry) and examined immediately using a NIKON Eclipse 80i upright fluorescent microscope equipped with a V2-A filter system (excitation 340 nm, barrier 514 nm). Images were captured using a NIKON DS-Qi1 digital camera and were processed using Image J.

Animals and Treatments

Heterozygous male C57BL/6J APC-Min (multiple intestinal neoplasia) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Heterozygous mice were used for these studies because homozygous mice die in utero. Mice in groups of 4–8 were fed either a high fat (11% fat) or low fat (1.2%) diet containing no Orlistat or Orlistat at 0.5 mg/g chow. Mice were started on the diets at either 50–60 days (Experiments 1 and 3) or 33–34 days

(Experiment 2) of age. Age at death varied from 104–229 days of age. Mice were housed in the Animal Care Facility at KCOM/ATSU and maintained according to the "Guide of the Care and Use of Laboratory Animals" from the National Research Council.

Recovery of Orlistat and Preparation of Test Diets

The contents of 4–6 Zenical capsules, each containing 120 mg of Orlistat, were pooled and extracted three times with 5 ml of 1:1 chloroform–methanol (v/v) using a Polytron-type homogenizer. The solvent from the pooled extracts recovered after centrifugation was evaporated under nitrogen. The residue was dried overnight to constant weight under high vacuum, and dissolved in ethanol to give a stock solution of 50 mg/ml. Mice in two studies were fed ground Purina Mouse Diet 5015 that contained 11% fat plus 0 (control) or 0.5 mg Orlistat/g chow. In a third study, mice were fed a customized powered diet (AIN-93M, Purina Mills TESTDIET, Richmond, IN) containing 0.2% fat, from saturated and monounsaturated fatty acids, to which we added 1% linoleic acid to guard against essential fatty acid deficiency. Orlistat was added at 0 (control) or 0.5 mg/g (test) to this very low fat diet.

HPLC Analysis of Orlistat

The HPLC method of Souri et al. [11] was used to confirm the concentration of Orlistat in chow and to estimate the distribution of Orlistat in the mouse gut following oral dosage. Samples of mouse chow (11% fat) with Orlistat and sections of mouse gut removed at 4 h after oral dosage were extracted with 1:1 chloroform–methanol, and the recovered lipids were fractionated by thin layer chromatography on Silica Gel G plates using a solvent of hexane: diethyl ether:glacial acetic acid (73:25:2). Orlistat, visualized by exposure to iodine vapor, migrated with an Rf of between 0.1 and 0.2. After sublimation of the iodine, the TLC zone containing Orlistat was recovered and extracted twice with 3 ml aliquots of methanol. The residue remaining after evaporation of the methanol was dissolved in several hundred microliters of acetonitrile:0.1% orthophosphoric acid in water (90:10), centrifuged and aliquots injected onto a Waters C18 reverse phase column (10 µm, 3.9 × 300 mm, 125 A µBondapak) eluted at 1 ml/min with the 90:10 acetonitrile-acidic water and absorbance monitored at 205 nm. Ten micrograms of Orlistat standard (injected in 100 µl) eluted at about 6 min.

Survival Statistical Analyses

To determine the effects of Orlistat on mouse survival, the end point analyzed was the difference in day of death between untreated (control) and Orlistat treated animals (test). The Kaplan–Meier procedure [18] was used to estimate the probability of surviving a given time. Since all untreated animals die prematurely (4–7 months) compared to wild type mice (1.5–2 years), even slight prolongation of life due to treatment can be recognized. Using a statistical computer program (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA) survival curves were generated by the Kaplan–Meier procedure [23] and analyzed by the Log-Rank (Mantel–Cox) and the Gehan–Wilcoxon Tests. The Log-Rank test is more powerful and gives equal weight to all time points.

Results

Potential use of Orlistat as an anticancer drug would likely be restricted to treatment of gastrointestinal tumors since it is essentially unabsorbed following oral dosage [2]. Therefore, we chose to examine the effects of Orlistat on a human derived gastric cancer cell line, NUGC-3 [24]. Viability of cells cultured for 48 h in serum deficient media, DM (0.10% serum), was decreased by more than 70% when continuously exposed to 25 µM Orlistat (Fig. 1). Near complete inhibition was seen at 100 µM. The dose response curve was markedly shifted to the right by including 10% serum in the media, called whole media (WM).

Although cell viability assays are typically performed following 48 h or more of continuous exposure to an inhibitor, we considered the possibility that the decreased viability observed at 48 h was due in part or total to cell injury induced soon after adding Orlistat rather than to a

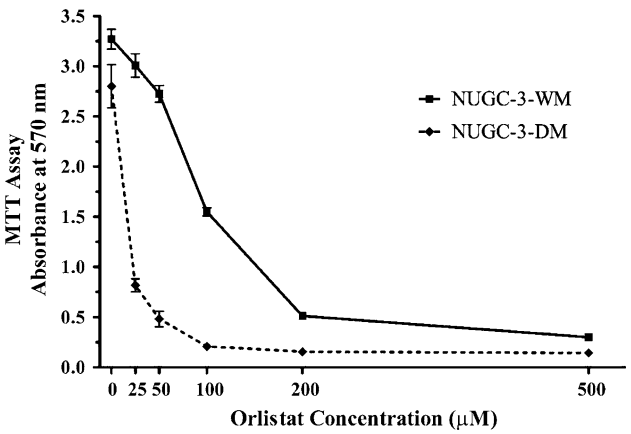

Fig. 1 Effect of Orlistat on the viability of NUGC-3 gastric tumor cells. Cells were cultured for 48 h in RMPI-1640 media containing 10% fetal calf serum (whole media, WM) or 0.10% serum (deficient media, DM) and varying concentrations of Orlistat. Cell viability was assessed by the MTT assay. Each point is the mean ± SEM of 6 cell wells

sustained accumulation of injury. Cells were incubated for 4, 8 or 12 h in DM containing 0 (control), 100 or 500 μM Orlistat, and then incubated to hour 48 in drug free media. Cell viability was significantly reduced from only 4-hour exposure to 500 μM Orlistat and an 8-hour exposure to 100 μM Orlistat (Fig. 2a). Four-hour exposure to 500 μM Orlistat decreased viability by about 65%, while 8-hour exposure to 100 μM decreased viability by about 40%. These brief exposure periods accounted for about two-thirds and one-half of the total decrease seen for cells cultured the entire 48 h in DM with 500 and 100 μM Orlistat, respectively. Addition of WM rather than DM to the NUGC-3 cell layers after 4 h exposure to 500 μM Orlistat and after 8 h exposure to 100 μM totally rescued the cells; that is, there was no decrease in viability compared to cells cultured in the absence of Orlistat measured at 48 h (Fig. 2b). However, there were limits to the capacity of WM to rescue Orlistat exposed cell. For example, 12 h of exposure to 500 μM Orlistat in DM committed the cells to detachment and disintegration.

Orlistat toxicity likely begins with inhibition of fatty acid synthesis. Incubation of NUGC-3 cells with 200 μM Orlistat for 4 h decreased incorporation of ^{14}C-acetate into total fatty acids by about 75% (Fig. 3a). We expressed effects on fatty acid synthesis as a decrease in the ratio of incorporation of ^{14}C-acetate into fatty acid to total sterol. Since Orlistat had no effect on sterol synthesis, this expression should account for potential differences in cell numbers from well to well. Not only does Orlistat inhibit fatty acid synthesis, but also once exposed to this drug the inhibition seems permanent. Fatty acid synthesis continued to be inhibited after Orlistat was removed from the media. NUGC-3 cells were incubated for 4 h in media containing 200 μM Orlistat, the cell layers were washed and then incubated an additional 4 h in Orlistat free media containing ^{14}C-acetate. Synthesis was still inhibited by 80% (Fig. 3b). This finding supports the idea that Orlistat binds irreversibly to the thioesterase domain of FAS [8]. Up regulation of FAS expression might be a means to circumvent the near total inhibition of fatty acid synthesis; however, FAS protein levels (assessed by Western blotting) were not increased following exposure of Orlistat (data not shown). Thus, once exposed to Orlistat fatty acid synthesis appears permanently inhibited. This would make the cells dependent upon uptake of lipids from the media as the only source of fatty acids to sustain membrane turnover and cell proliferation.

As shown (Fig. 2), there was a narrow window of opportunity to rescue NUGC-3 cell from permanent injury and death when cultured in DM containing Orlistat. The response of the cells to this nutritional stress, no source of fatty acids, was extremely rapid induction of autophagy (Fig. 4). The autophagic response was Orlistat dose dependent, peaked at one-half hour of incubation and returned to baseline by 2 h. Thus, it seems unlikely that autophagy accounts for the irreversible injury and consequent cell

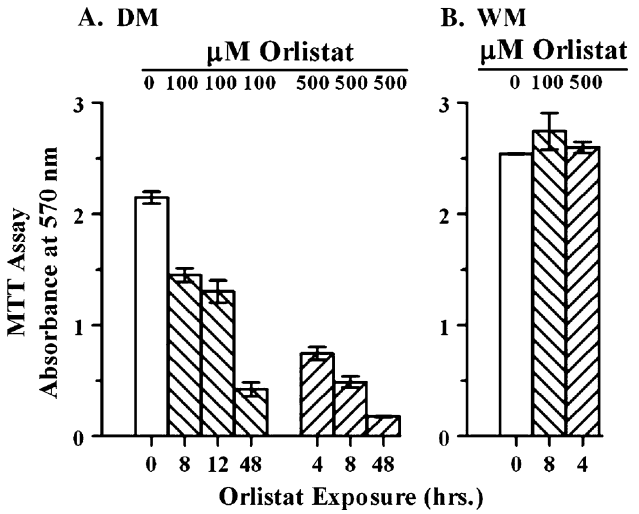

Fig. 2 Early effects of Orlistat on cell viability. **a** NUGC-3 cell layers were incubated in serum deficient media containing 0, 100 or 500 μM Orlistat. After 4, 8 or 12 h of incubation the media was replaced with Orlistat free DM and the incubation continued to hour 48 at which time cell viability was measured. Controls were cultured for 48 h in Orlistat free DM. The "48" hour groups were cells incubated with either 100 or 500 μM Orlistat-DM. **b** Controls are cells cultured for 48 h in Orlistat free WM. Cells were incubated for 4 h in DM containing 500 μM Orlistat or 8 h with 100 μM Orlistat and then switched to Orlistat free WM and the culture continued to hour 48 at which time viability was measured

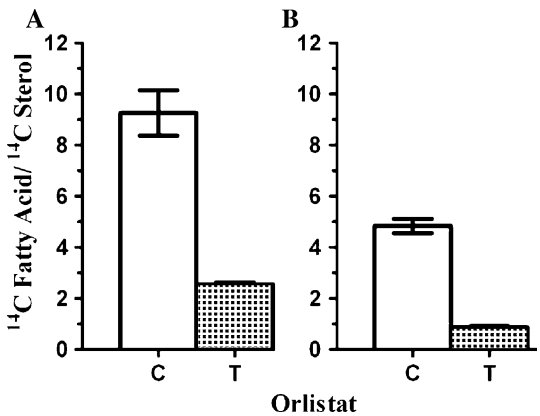

Fig. 3 Irreversible inhibition of fatty acid synthesis by Orlistat in NUGC-3 tumor cells. **a** Subconfluent cell layers in 96 well dishes were incubated for 4 h in serum free RMPI-1640 media containing 1 μCi of $^{1\text{-}14}$C-acetate and 0 (Control *C*) or 200 μM Orlistat (Treated *T*). Total fatty acids and sterols recovered after saponification were separated by TLC, fractions isolated, the ^{14}C-content measured and results expressed as the ratio of incorporation into fatty acids to sterols. Each value is the mean ± SEM of 5 cell layers. **b** Cell layers were incubated for 4 h with 0 or 200 μM Orlistat in the absence of ^{14}C-acetate; the media was then replaced with Orlistat free media containing the ^{14}C-acetate and incubated for a second 4-hour interval. Values are the mean ± SEM of five cell layers

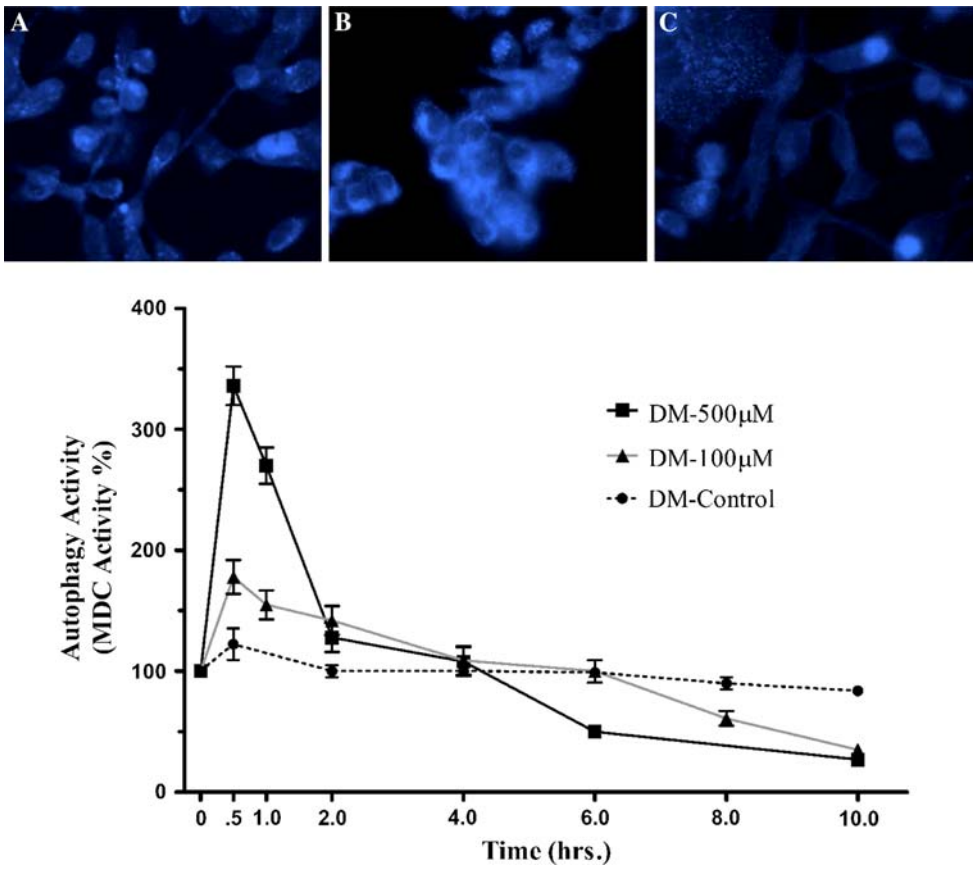

Fig. 4 Rapid induction and disappearance of the autophagic response to Orlistat. Fluorescence microscopy showing autophagic vesicles in NUGC-3 cells unexposed to Orlistat (**a**), exposed to 500 μM Orlistat for 30 min (**b**) or 6 h (**c**). The graph shows the time course of autophagic response to varying concentrations of Orlistat. Values are the mean ± SEM of 3 cell layers. Differences in cell numbers between wells normalized using ethidium bromide [21]

death seen with longer-term exposure. Rather, cell death could be due to induction of apoptosis. The onset and progression of apoptosis paralleled the loss of cell viability. Apoptosis was seen after 8 h exposure to 100 μM Orlistat in DM (Fig. 5e), but not at 4 h (Fig. 5c). Loss of cell viability occurred after 8 but not 4 h exposure to 100 μM (Fig. 2 a). At the higher drug concentration (500 μM), apoptosis was evident after 4 h of incubation (Fig. 5d) and markedly increased by the 8 h of drug exposure (Fig. 5f). The progression of apoptosis over the 4–8 h interval was largely halted by exchanging the Orlistat-DM media for drug-free WM at hour four (Fig. 5g). This parallels the time-interval boundaries for rescue of NUGC-3 cell viability from exposure to 500 μM Orlistat (Fig. 2).

Applying the information garnered from in vitro experiments, the potential of Orlistat to inhibit in vivo gastrointestinal cancer was evaluated by examining the effect of orally administered Orlistat on the survival of APC-Min mice that spontaneously develop a fatal gastrointestinal cancer. Mice were fed a high fat (11%) or low fat (1.2%) diet containing 0 or 500 mg Orlistat/kg chow. Each mouse weighed approximately 25 g and consumed an average of 3 g of chow per day, resulting in delivery of about 60 mg of Orlistat/kg body weight. The fecal fat content of Orlistat treated mice increased 4-to-6-fold compared to untreated control mice when the animals were fed chow containing 11% fat (Table 1). In animals fed chow containing 1.2% fat, fecal fat increased about 2.5-fold in Orlistat treated mice versus untreated controls (Table 1). The increased fecal fat obviously reflects the inhibition of gastric lipases by the drug.

Orally administered Orlistat moves quickly through the GI tract. Four hours after giving a single oral 50 mg/kg dose of Orlistat (in aqueous emulsion) by gavage, the drug was detected almost exclusively in the large intestines (Fig. 6). Similar results were seen in a replicate experiment. This observation is consistent with GI transit times in the mouse being 4–6 h [25].

Considering the browsing feeding pattern of mice, the gut should receive multiple exposures to Orlistat during the day. The 500 mg of Orlistat/kg of chow corresponds to about 1 mM (MW = 497.5), a concentration in great excess of that needed to inhibit the viability of cultured, human–derived, NUGC-3 gastrointestinal cancer cells (Fig. 1). Fifty μM Orlistat in serum deficient media (0.1% serum) and 200 μM Orlistat in whole media (10% serum) both inhibited cell viability by about 80% (Fig. 1). Similar results were seen in replicate experiments.

Three experiments were conducted to evaluate the effect of orally administered Orlistat on survival times of the

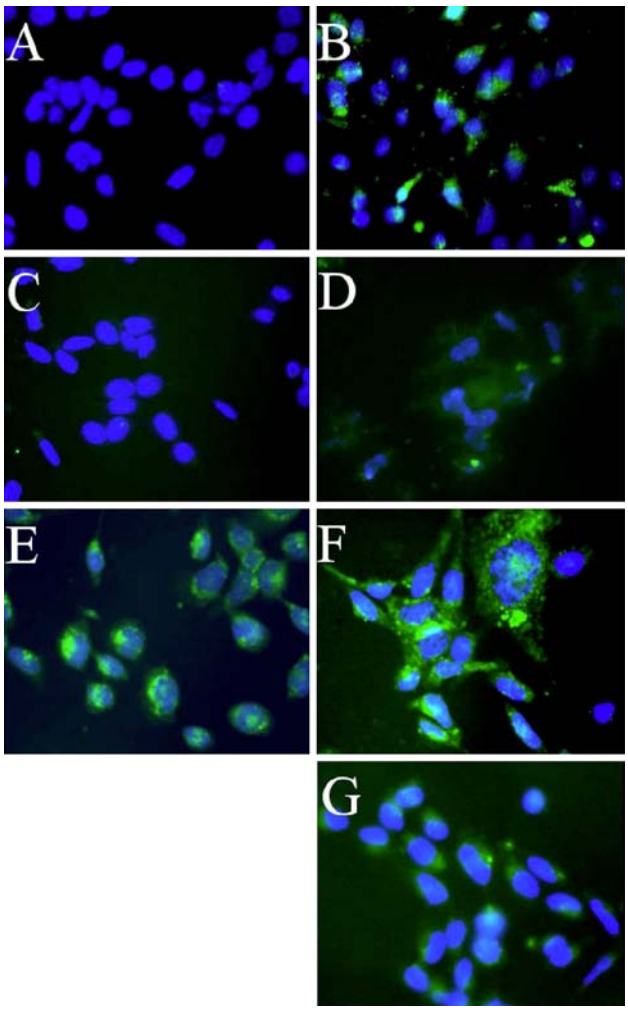

Fig. 5 Antagonism of Orlistat induced apoptosis. Images show overlap fluorescence of apoptotic caspase stained (*green*) and nucleic acid stained (*blue*) cells**.** All cell layers, except **b** and **g**, were incubated in DM. **a** Control. **b** Staurosporine, 500 nM in WM for 3 h. **c** Orlistat, 100 μM for 4 h. **d** Orlistat, 500 μM for 4 h. **e** Orlistat, 100 μM for 8 h. **f** Orlistat, 500 μM for 8 h. **g** Cell layers were incubated for 4 h with 500 μM Orlistat, the media was replaced with Orlistat free WM and the incubation continued an additional 4 h. These results are representative of triplicate experiments (color figure online)

APC-min mice (Fig. 7). Median survival times were increased 7–10% (25.5 and 9 days, experiments 1 and 2, respectively) in Orlistat fed mice (Fig. 7 top and middle panels). The difference was statistically significant by the log-rank test ($P = 0.047$) and the Wilcoxon test ($P = 0.0318$) in experiment 1 and was not significant in experiment 2 (Table 2). Experiment 3, using a low fat (1.2%) diet produced no significant results (Table 2), however, control mice fed the low fat diet produced a greater median survival time than their treated counterparts (Table 1).

Table 1 Effect of Orlistat on fecal fat content and median survival times

Experiment	Group	Fecal fat content[a] (mg fat/g dry feces)	Starting age (days)	Median survival (days)
1 High fat	Control	45.3	50–60	147
	Treated	197.8		172.5
2 High fat	Control	63.7	33–40	122
	Treated	388.8		131
3 Low fat	Control	22.4	58	194.5
	Treated	52		136

[a] Controls were fed diets with either 11% high fat (Experiment 1 and 2) or 1.2% low fat (Experiment 3). Treated mice received the same diet but containing 0.5 mg Orlistat/g chow. Feces was collected from the cages of the various groups

Discussion

As stated above, cancer cells can place a great demand on FAS to supply the fatty acids required for membrane turnover and cell proliferation. NUGC-3 cells cultured in serum deficient media (0.1% serum) grew as well as when cultured in media containing 10% serum, indicating that lipogenesis can sustain growth. In contrast to cancer cells, viability of a normal cell line (bovine lens epithelial) decreased by about 50% when cultured in serum deficient media, indicating a major reliance on extracellular lipids (Fig. 8). Also, the normal bovine lens epithelial cells were virtually resistant to the growth inhibition mediated by high concentrations of Orlistat in WM, indicating little reliance on fatty acid synthesis (Fig. 8). The general toxicity of Orlistat is also addressed by this data. Since the viability of lens epithelial cells cultured in WM containing no Orlistat and high levels of Orlistat (200 and 500 μM) were similar, Orlistat has no apparent toxicity to these cultured cells independent of its effect on FAS. This would appear to also include toxicity due to inhibition of triacylglycerol lipases, the basis of its use as an anti-obesity drug [7].

Perhaps the major findings of this work are the speed with which Orlistat can insult the cultured cancer cells and the capacity of whole media to rescue the cells from this insult, at least up to a point. The rapid untoward effects are attributed to near total restriction of fatty acids due to inhibition of synthesis coupled with the absence of media lipids. Because membrane phospholipids are highly dynamic and can turnover with half-lives in the minutes [26], a block in the availability of fatty acids could rapidly compromise membrane integrity. In view of this rapid turnover and because the restriction of fatty acids constitutes nutritional stress, it is not surprising that autophagy was activated within minutes of adding Orlistat. However, it was surprising that the autophagic response was over

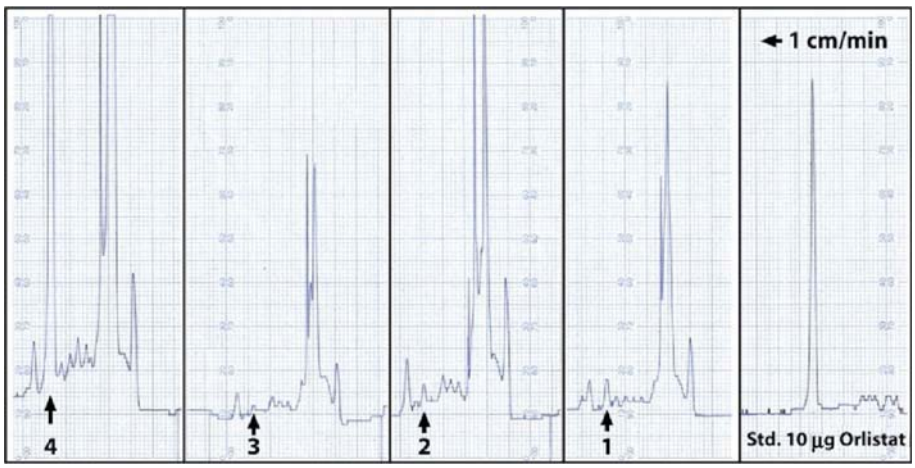

Fig. 6 Distribution of Orlistat in the gastrointestinal tract estimated by HPLC analysis. Relative concentration of Orlistat in the GI tract at 4 h after a mouse was given (by gavage) 50 mg/kg of Orlistat in aqueous emulsion. Nearly all of the drug was found in the large intestine and colon. Similar results were seen in a replicate experiment. The Orlistat standard (10 µg injected in 100 µl) eluted at about 6 min. Zones: *1* Stomach, *2* Upper small intestine, *3* Lower small intestines, *4* Large intestines and colon

within 2 h. This could reflect that there is a limited pool of cellular substrates for the autophagic response. The extent to which the autophagy damaged the cells is unclear, but it is clear that apoptosis was activated by 4–8 h of culture, depending on the concentration of Orlistat. Early signs of apoptosis, cellular blebbing, were observed in as little as 4 h following treatment with 500 µM Orlistat substituted DM and 12 h exposure to 100 µM Orlistat substituted DM. The apoptosis is linked to the nutritional deficiency since adding a source of fatty acids to the media suppressed its progression and reversed the signs of cellular blebbing. Apparently the cells can recover, re-attain normal viability, from initiated apoptosis. However, there is clearly a threshold of apoptosis inducing cell injury beyond which the cells cannot recover.

Knowles and coworkers [9] describe in a paper that the apoptosis induced in mammary and prostate cell tumor lines by Orlistat was dependent on inhibition of FAS but independent of fatty acid availability. This paradox is explained by a pleiotropic consequence of inhibiting FAS that involves up-regulation of a stress responsive gene, DDIT4, which inhibits the mTOR pathway. Our findings with the NUGC-3 gastric tumor cells are more consistent with apoptosis arising from nutritional stress due to decreased availability of fatty acids. Addition of lipid-containing media to NUGC-3 cells exposed to Orlistat for 4 h halted progression of apoptosis over the next 4 h. Even though Orlistat was removed from the media in the second incubation, FAS continued to be inhibited (Fig. 2b). Thus, in the face of continued inhibition of FAS, whole media alone (lipids) halted apoptosis. The differences between our findings and those of Knowles et al. [9] could be related to differences in the duration of incubation with Orlistat. Eight hours exposure of NUGC-3 cells to 100 µM Orlistat clearly induced apoptosis (Fig. 5e). Perhaps a higher level of stress develops with 24 and 48 h of continuous exposure that activated the DDIT4 initiated apoptosis. Although we used higher concentrations of Orlistat, their observations at 50 µM and ours at 100 µM should be similar. Our use of a gastric cell line may also have contributed to differences between our findings. As stated above, if Orlistat is to have a role in treating cancer it would be most likely for gastrointestinal tumors that could come in direct contact with the orally administered drug.

The greater sensitivity to inhibition of NUGC-3 cells in serum deficient media was likely due to the low lipid content of this media, since viability was greater in WM. This observation is consistent with FAS being Orlistat's target in cancer cells and Orlistat being more toxic to the cancer cells when they are wholly dependent on FAS to supply the fatty acids needed for phospholipid synthesis and, therefore, membrane formation.

Because increased cell death was seen in cultured cancer cells treated with Orlistat in DM, we applied this information to one of our in vivo experiments. Orlistat was fed in a very low fat diet in experiment 3. Animals were 58 days of age at the start. Previous work in our laboratory had showed that when added to a lipid emulsion, Orlistat partitioned into the lipid phase (data not shown). Given this result, we reasoned that Orlistat might similarly partition into the increased intestinal fat content generated by inhibition of lipases, and therefore, be less available to the tumors. Therefore, Orlistat was administered in a very low fat diet in the third experiment with the goal of enhancing drug exposure to gastrointestinal tumors. No statistically significant differences were seen between control and Orlistat treated groups (Fig. 7 bottom panel, Table 2). Although no significant difference was observed, the

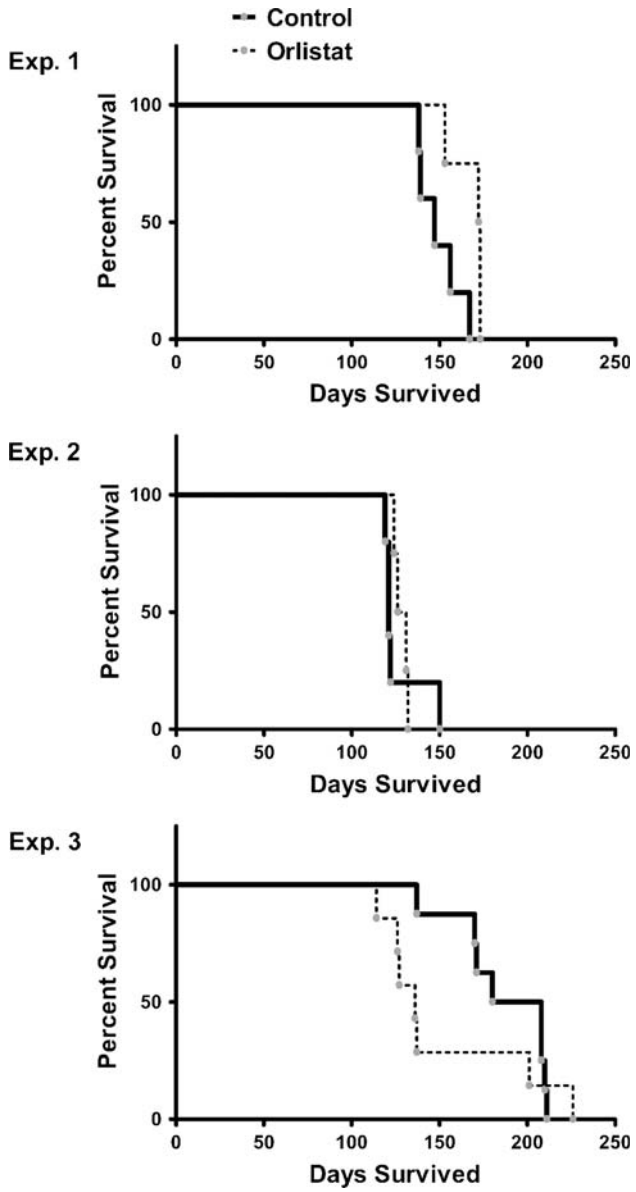

Fig. 7 Survival curves of control and Orlistat treated APC-min/+ mice. In experiments 1 and 2 mice were fed an 11% fat diet containing no Orlistat or 0.5 mg Orlistat/g chow. In experiment 3 mice were fed a 1.2% fat diet with and without 0.5 mg Orlistat/g chow

Table 2 Statistical analyses of experiments

	Experiment 1	Experiment 2	Experiment 3
Log-Rank (Mantel–Cox) Test			
Chi Square	4.609	0.3990	0.8191
P Value	0.0318	0.5276	0.3654
Gehan–Breslow–Wilcoxon Test			
Chi Square	3.938	2.201	3.530
P Value	0.0472	0.1379	0.0602

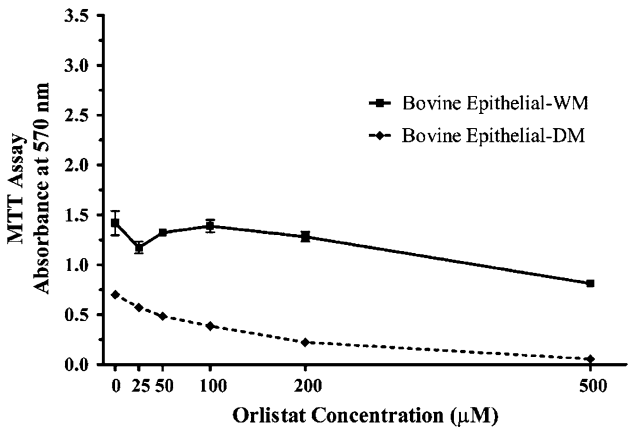

Fig. 8 Effect of Orlistat on the viability of bovine lens epithelial cells. Cells in 96 well plates were cultured for 48 h with varying concentrations of Orlistat added in either serum deficient DMEM (0.1% calf serum), DM, or DMEM containing 10% serum, WM. Cell viability was measured at hour 48 by the MTT assay. Values are mean ± SEM of 4 wells

median survival time for untreated mice fed the low fat diet exceeded that of the Orlistat treated group. This data suggest that limiting exogenous sources of fatty acids via diet restrictions may offer potential in treating gastrointestinal cancers. However, a significant difference was seen when Orlistat was supplemented into the high fat diet. This data suggest that when supplemented into a high fat diet, Orlistat produced more beneficial results than supplemented into an already fat limited diet.

In conclusion, Orlistat appeared to slightly prolong the survival of mice with a fatal-genetic gastrointestinal cancer. There was no evidence that Orlistat accelerated death in these tumor-bearing animals. If the findings are applicable to humans, they support the idea that Orlistat inhibits this type of cancer. Long-term survival studies in rats and other mice strains are needed to further evaluate the potential of Orlistat to inhibit GI cancer.

Acknowledgments This work was funded by grants from the A.T. Still University of Health Sciences.

References

1. Kuhajda FP (2006) Fatty acid synthase and cancer: new application of an old pathway. Cancer Res 66:5977–5980
2. Kuhajda FP (2000) Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. Nutrition 16:202–208
3. Milgraum LZ, Witters LA, Pasternack GR, Kuhajda FP (1997) Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. Clin Cancer Res 3:2115–2120
4. Pizer ES, Pflug BR, Bova GS, Han WF, Udan MS, Nelson JB (2001) Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. Prostate 47:102–110

5. Rossi S, Graner E, Febbo P, Weinstein L, Bhattacharya N, Onody T, Bubley G, Balk S, Loda M (2003) Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. Mol Cancer Res 1:707–715
6. Pizer ES, Chrest FJ, DiGiuseppe JA, Han WF (1998) Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. Cancer Res 58:4611–4615
7. (2006) PDR guide to drug interactions, side effects, and indications. Thomson PDR, Montvale, NJ
8. Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW (2004) Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Res 64:2070–2075
9. Knowles LM, Axelrod F, Browne CD, Smith JW (2004) A fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating Skp2. J Biol Chem 279:30540–30545
10. Menendez JA, Vellon L, Lupu R (2005) Orlistat: from anti-obesity drug to anticancer agent in Her-2/neu (erbB-2)-over-expressing gastrointestinal tumors? Exp Biol Med (Maywood) 230:151–154
11. Souri E, Jalalizadeh H, Kebriaee-Zadeh A, Zadehvakili B (2007) HPLC analysis of orlistat and its application to drug quality control studies. Chem Pharm Bull (Tokyo) 55:251–254
12. Jacoby RF, Seibert K, Cole CE, Kelloff G, Lubet RA (2000) The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. Cancer Res 60:5040–5044
13. Chiu CH, McEntee MF, Whelan J (1997) Sulindac causes rapid regression of preexisting tumors in Min/+mice independent of prostaglandin biosynthesis. Cancer Res 57:4267–4273
14. Moser AR, Dove WF, Roth KA, Gordon JI (1992) The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J Cell Biol 116:1517–1526
15. Taketo MM (2006) Mouse models of gastrointestinal tumors. Cancer Sci 97:355–361
16. Hitchener WR, Cenedella RJ (1985) Absolute rates of sterol synthesis estimated from [3H]water for bovine lens epithelial cells in culture. J Lipid Res 26:1455–1463
17. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:936–942
18. Sexton PS, Neely AR, Cenedella RJ (2004) Distribution of caveolin-1 in bovine lens and redistribution in cultured bovine lens epithelial cells upon confluence. Exp Eye Res 78:75–82
19. Biederbick A, Kern HF, Elsasser HP (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 66:3–14
20. Munafo DB, Colombo MI (2001) A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114:3619–3629
21. Darzynkiewicz Z, Bedner E, Smolewski P, Lee BW, Johnson GL (2002) Detection of caspases activation in situ by fluorochrome-labeled inhibitors of caspases (FLICA). Methods Mol Biol 203:289–299
22. Grabarek J, Amstad P, Darzynkiewicz Z (2002) Use of fluorescently labeled caspase inhibitors as affinity labels to detect activated caspases. Hum Cell 15:1–12
23. Daniel WW (2005) Biostatistics: a foundation for analysis in the health sciences. Wiley, Hoboken, NJ
24. Akiyama S, Amo H, Watanabe T, Matsuyama M, Sakamoto J, Imaizumi M, Ichihashi H, Kondo T, Takagi H (1988) Characteristics of three human gastric cancer cell lines, NU-GC-2, NU-GC-3 and NU-GC-4. Jpn J Surg 18:438–446
25. Garcia SB, Barros LT, Turatti A, Martinello F, Modiano P, Ribeiro-Silva A, Vespucio MV, Uyemura SA (2006) The anti-obesity agent Orlistat is associated to increase in colonic preneoplastic markers in rats treated with a chemical carcinogen. Cancer Lett 240:221–224
26. Hao M, Maxfield FR (2000) Characterization of rapid membrane internalization and recycling. J Biol Chem 275:15279–15286