COMMENTS

Orlistat: From Antiobesity Drug to Anticancer Agent in Her-2/*neu* (*erb*B-2)-Overexpressing Gastrointestinal Tumors?

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The U.S. Food and Drug Administration (FDA)-approved antiobesity drug Orlistat (marketed by Roche [Basel, Switzerland] as Xenical) is a semisynthetic derivative of lipstatin that irreversibly inhibits pancreatic and gastric lipases within the gastrointestinal (GI) tract (1). Interestingly, an intense public interest has been generated by the characterization of Orlistat as a potent inhibitor of prostate and breast tumor growth (2). Mechanistically, it has been demonstrated that Orlistat exhibits antiproliferative and antitumor properties toward prostate and breast cancer cells by virtue of its ability to block the lipogenic activity of fatty acid synthase (FAS) (2, 3). Fatty acid synthase is the key metabolic muti-enzyme that is responsible for the terminal catalytic step in the *de novo* fatty acid biosynthesis (4). Upregulation of FAS expression and activity, an anabolic-energy-storage pathway largely considered of minor importance in humans, is a very early and nearly universal neoplastic marker that positively correlates with aggressive behaviors and poorer clinical outcome prognoses in many human cancers (5). Interestingly, we recently demonstrated that cancer-associated FAS, which to date appeared to be part of a growth factor-driven pleiotropic change in the genetic program controlling lipogenesis, actively contributes to the cancer phenotype by regulating the expression, activity, and cellular localization of the Her-2/*neu* (*erb*B-2) oncogene (6), a master player in the etiology and aggressive behavior of several cancers (7).

Because Orlistat possesses extremely low oral bioavailability, a novel formulation will be required for treating tumors such as prostate or breast carcinomas. However, we hypothesized that Orlistat-induced inhibition of FAS activity may represent a valuable therapeutic strategy to test in GI tumors overexpressing Her-2/*neu* oncogene, that is, associated with approximately one fourth of all GI tract malignancies (8). To test this hypothesis, we employed the highly metastatic and Her-2/*neu*-overexpressing NCI-N87 stomach carcinoma cell line. Micromolar concentrations of Orlistat (up to 40 μM) induced dose-dependent antiproliferative effects against NCI-N87 cells when relative cell numbers were determined using a 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT)-based characterization of metabolically viable cells (Fig. 1a). Cell cycle analyses revealed that Orlistat exposure induced a complete loss of G_2-M cell population with a concomitant increase of cells in G_1 by 48 hrs. This G_1 blockade of Orlistat-treated NCI-N87 cells was accompanied with significant increases

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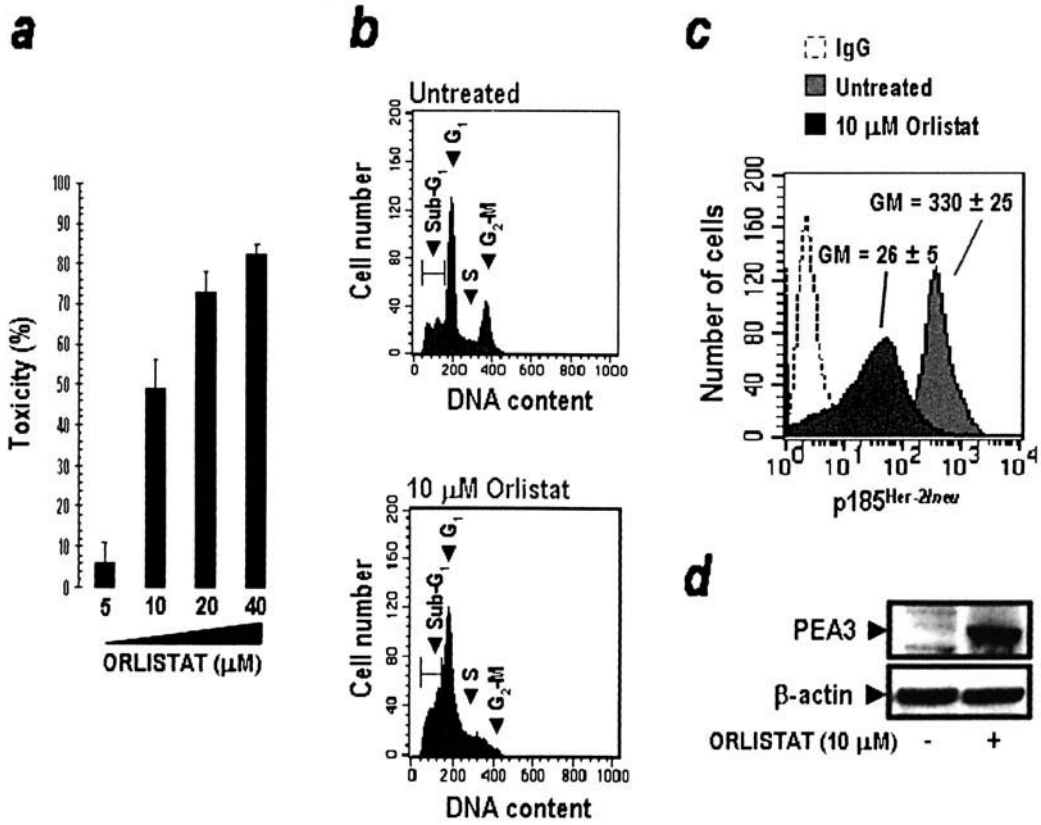

Figure 1. (a) Orlistat-induced blockade of fatty acid synthase (FAS) activity inhibits gastrointestinal (GI) carcinoma cell proliferation. The figure shows the percentage growth inhibition of Her-2/*neu*-overexpressing NCI-N87 stomach cancer cells by increasing concentrations of the statin Orlistat for 96 hrs, calculated from 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT)-based cell viability assays, and expressed as a percent growth inhibition compared with cells grown in the presence of ethanol as control (= 0% toxicity). Values shown are means (columns) ± SD (bars) from triplicate wells and are representative of repeated experiments. (b) Orlistat blocks GI cell cycle progression. After 48 hrs of treatment with 10 μ*M* Orlistat, adherent and detached cells were collected after trypsin detachment, washed in phosphate-buffered saline (PBS) and centrifuged at 1500 rpm. Cells were resuspended at 2×10^6 cells/ml in PBS and fixed in ice-cold 70% ethanol for at least 24 hrs. Fixed cells were centrifuged at 1500 rpm, and each sample resuspended in propidium iodide (PI) stain buffer (0.1% Triton X-100, 200 μg of DNase-free RNase A, 20 μg of PI) in PBS for 30 mins. After staining, samples were analyzed using a FACScalibur (Becton Dickinson, San Diego, CA) and ModFit LT (Verity Software). (c) The statin Orlistat suppresses $p185^{Her\text{-}2/neu}$ oncoprotein expression in Her-2/*neu*-overexpressing GI carcinoma cells. After 48 hrs of treatment with 10 μ*M* Orlistat, the specific surface expression of Her-2/*neu*-coded $p185^{Her\text{-}2/neu}$ oncoprotein in NCI-N87 stomach carcinoma cells was determined by flow cytometry by measuring the binding of a mouse anti-Her-2/*neu* antibody directed against the extracellular domain of Her-2/*neu* (clone Ab-5; Oncogene Research Products, San Diego, CA). The flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson) equipped with Cell Quest Software (Becton Dickinson). The mean fluorescence signal associated with cells for labeled $p185^{Her\text{-}2/neu}$ was quantified using the Geo Mean (GM) fluorescence parameter provided with the software. All observations were confirmed by at least three independent experiments. The data are presented as means ± SD. (d) Orlistat-induced blockade of FAS activity upregulates PEA3 expression in GI carcinoma cells. NCI-N87 cells were treated for 48 hrs with either ethanol (volume per volume) or 10 μ*M* Orlistat. Total protein (50 μg) was resolved by SDS-PAGE and subjected to immunoblot analyses for PEA3 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were reprobed for β-actin (Santa Cruz Biotechnology) to control for protein loading and transfer. Results are representative of three independent experiments.

(~3-fold) of cells in the emerging sub-G_1 (apoptotic) cell population (Fig. 1b). Moreover, flow cytometric analyses established the ability of Orlistat (10 μ*M*, 48-hr exposure) to dramatically decrease (>90% reduction) the expression levels of Her-2/*neu*-coded $p185^{Her\text{-}2/neu}$ oncoprotein in NCI-N87 cells (Fig. 1c). To gain additional insight into the molecular mechanisms underlying the downregulation of Her-2/*neu* induced by Orlistat, we examined the expression of Her-2/*neu* regulator PEA3 following Orlistat-induced blockade of FAS activity in NCI-N87 cells. The DNA-binding protein PEA3, a member of the *Ets* transcription factor family, specifically targets a DNA sequence on the Her-2/*neu* promoter and downregulates its promoter activity, thus suppressing Her-2/*neu* overexpression and inhibiting tumorigenesis (9). Interestingly, a significant accumulation of PEA3 protein was observed in immunoblotting analyses of Orlistat-treated NCI-N87 cells (Fig. 1d). We next tested whether Orlistat-induced accumulation of PEA3 correlated with a transcriptional response of Her-2/*neu* gene. When a luciferase reporter gene driven by the Her-2/*neu* promoter (pNulit) was transiently transfected into the NCI-N87 stomach carcinoma cell line, Orlistat exposure for 48 hrs was found to significantly repress the promoter activity of Her-2/*neu* by ~60% (Fig. 2a, left panel). When we characterized Orlistat's effects on the activity of a Her-2/*neu* promoter bearing a mutated PEA3 binding sequence

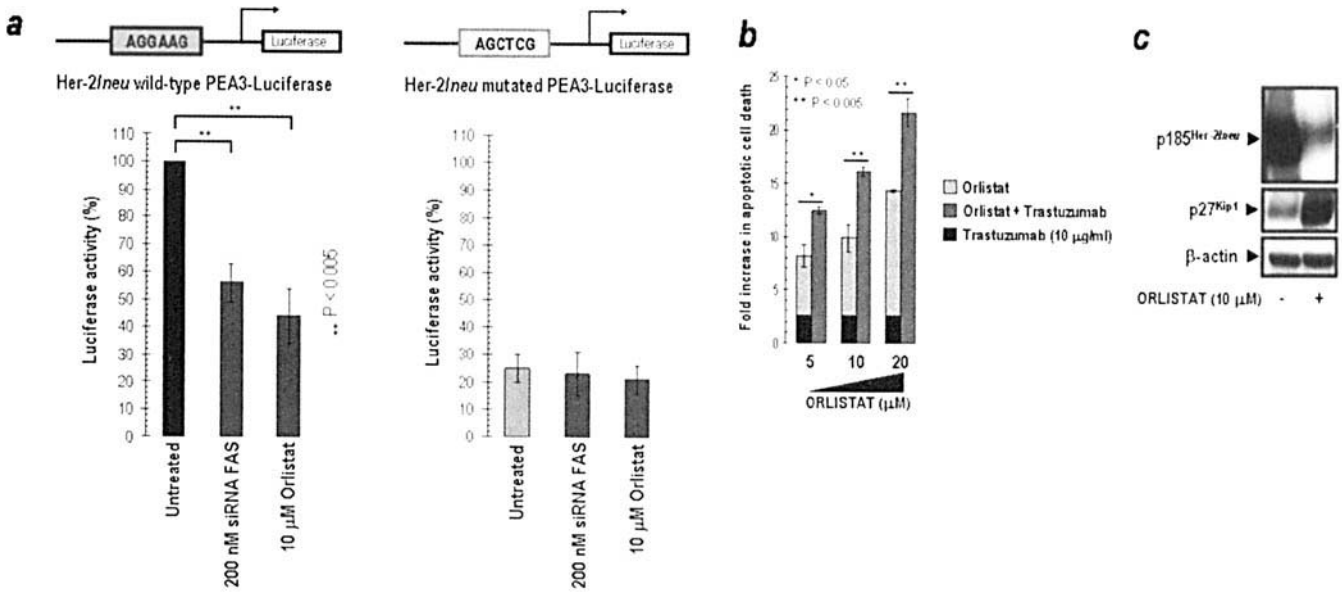

Figure 2. (a) Orlistat-induced fatty acid synthase (FAS) blockade represses Her-2/*neu* promoter activity through a positive regulatory DNA motif. Luciferase reporter gene driven by either wild-type (left panel) or the PEA3-mutated (right panel) Her-2/*neu* promoter was measured after 48 hrs treatment with either 10 μ*M* Orlistat or 200 n*M* siRNA targeting FAS gene (the double-stranded siRNA FAS was CCCUGAGAUCCCAGCGCUGdTdT, sense; and CAGCGCUGGGAUCUCAGGGdTdT, antisense; see Ref. 6) by following the manufacturer's (Promega, Madison, WI) instructions and using a Victor$^2_{1420}$ Multilabel Counter (Perkin-Elmer Life Sciences, Wellesley, MA). The activity of the wild-type promoter in untreated control cells was defined as 100%. Values shown are means (columns) ± SD (bars) from triplicate wells and are representative of repeated experiments. (b) Concurrent exposure to Orlistat and trastuzumab (Herceptin) synergistically promotes apoptotic cell death in NCI-N87 cells. The induction of cell death by exposure of NCI-N87 cells to Orlistat and trastuzumab was assessed using the Cell Death Detection ELISA^{PLUS} kit obtained from Roche Molecular Biochemicals (Indianapolis, IN). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after cell death. Briefly, cells (7.5×10^3/well) were grown in 96-well plates and treated, in duplicates, for 72 hrs with the indicated doses of Orlistat, trastuzumab, or Orlistat *plus* trastuzumab. The induction of cell death was evaluated using cytosolic fractions obtained from pooled adherent and floating cells (obtained by centrifugation at 200 *g* for 10 mins) by assessing the enrichment of nucleosomes in the cytoplasm using antihistone biotin and anti-DNA peroxidase antibodies (room temperature for 2 hrs) and determined exactly as described in the manufacturer's protocol. After three washes, the peroxidase substrate was added to each well and the plates were read at 405 nm at multiple time intervals. The enrichment of histone-DNA fragments in treated cells was expressed as a fold increase in absorbance as compared with control (vehicle-treated) cells. The Student's *t* test was used to evaluate the statistical significance of mean values. Statistical significance levels were $P < 0.05$ (denoted as $*$) and $P < 0.005$ (denoted as $**$). All *P* values are two-tailed. (c) Orlistat-induced blockade of FAS activity upregulates p27^{Kip1} expression in gastrointestinal carcinoma cells. NCI-N87 cells were treated for 48 hrs with either ethanol (volume per volume) or 10 μ*M* Orlistat. Total protein (50 μg) was resolved by SDS-PAGE and subjected to immunoblot analyses for either p185$^{Her\text{-}2/neu}$ (clone Ab-3; Oncogene Research Products, San Diego, CA) or p27^{Kip1}. Blots were reprobed with for β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) to control for protein loading and transfer. Results are representative of three independent experiments.

(5′-AGGAAG-3′ to 5′-AGCTCG-3′), we concluded that the intact PEA3 binding site on the Her-2/*neu* promoter is required for the Orlistat-mediated transcriptional repression of Her-2/*neu* gene expression in NCI-N87 cells. Thus, the luciferase reporter gene driving the PEA3 site-mutated sequence was not subject to negative regulation by Orlistat (Fig. 2a, right panel). Importantly, RNA interference (RNAi)-mediated silencing of the FAS expression following transfection with sequence-specific double-stranded RNA oligonucleotides targeting FAS gene (6) similarly repressed Her-2/*neu* promoter activity in a PEA3-dependent manner (Fig. 2a), thus ruling out a role for non-FAS Orlistat-mediated effects in Her-2/*neu* downregulation. These findings, altogether, constitute an independent strategy to confirm our hypothesis that FAS inhibition is accompanied by the specific suppression of Her-2/*neu* oncogene expression in cancer cells (6).

To evaluate whether Orlistat-induced transcriptional repression of Her-2/*neu* oncogene actively modulated the efficacy of the anti-Her-2/*neu* humanized monoclonal antibody trastuzumab, NCI-N87 cells were exposed to increasing concentrations of Orlistat in the presence or absence of 10 μg/ml trastuzumab, cell death was measured by an enzyme-linked immunosorbent assay (ELISA) that detects DNA-histone fragmentation, and the *x*-fold increase in apoptosis-related cell death was calculated by comparing the ELISA optical density readings of treated samples, with the values of the untreated cells as 1.0. Remarkably, Orlistat-induced downregulation of Her-2/*neu* oncogene expression in NCI-N87 cells synergistically worked with trastuzumab to promote apoptosis in this GI cancer model (Fig. 2b). Because the cyclin-dependent kinase inhibitor (CDK_i) p27^{Kip1} plays a key role in the onset and progression of Her-2/*neu*-related carcinomas (10), low p27^{Kip1} expression is regarded as an important adverse prognostic factor in GI cancers (11), and trastuzumab resistance may be associated with decreased p27^{Kip1} levels and may be susceptible to treatments that induce p27^{Kip1} expression

(12), we finally examined the expression levels of $p27^{kip1}$ following Orlistat treatment. NCI-N87 cells exposed to 10 μM Orlistat over a 48-hr time course demonstrated a dramatic accumulation of the $p27^{Kip1}$ protein concomitantly with Orlistat-induced downregulation of $p185^{Her\text{-}2/neu}$ expression (Fig. 2c).

Although caution must be applied when extrapolating *in vitro* results into clinical practice, the significant cytotoxicity, the blockade of cell cycle progression, and the dramatic repression of Her-2/*neu* expression that occur following Orlistat treatment strongly suggest that this selective lipase inhibitor used for treating obesity may represent a suitable drug candidate for treating Her-2/*neu*-overexpressing GI carcinomas through its ability to block tumor-associated FAS hyperactivity. Moreover, Orlistat supra-additively interacts with trastuzumab-based anti-Her-2/*neu* immunotherapy, which is associated with a PEA3-dependent transcriptional repression of Her-2/*neu* gene and an increase in $p27^{Kip1}$ protein levels, thus providing appropriate surrogate markers to follow treatment efficacy *in vivo*. Although the efficacy of Orlistat in a wider panel of GI carcinoma cell lines expressing different amounts of Her-2/*neu* remains to be elucidated, we recently observed that MCF-7 breast cancer cells, which express physiological amounts of Her-2/*neu* oncogene, became hypersensitive to Orlistat when they were engineered to overexpress Her-2/*neu* (data not shown). These findings, altogether, support the concept that inhibition of FAS activity may provide a new molecular avenue for chemotherapeutic prevention and/or treatment of Her-2/*neu*-dependent carcinomas (6, 13). Considering that studies evaluating Orlistat's long-term effects have begun to emerge (14), future analyses of the incidence and clinical outcome of GI carcinomas in a representative obese population earlier receiving Orlistat treatment may illustrate further the chemopreventive effects of this statin to GI tumors.

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