The peptide-hormone glucagon-like peptide-1 activates cAMP and inhibits growth of breast cancer cells

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Abstract The incretin hormone glucagon-like peptide (GLP)-1 is secreted from intestinal L cells in response to food intake, and promotes insulin secretion and pancreatic β-cell proliferation. Reduced GLP-1 levels are observed in obesity and type 2 diabetes mellitus (T2DM) and are associated with reduced insulin secretion and increased insulin resistance. GLP-1 mediates its activities through activation of a G-protein coupled receptor, which is expressed in the pancreas, as well as other tissues. Long-acting GLP-1 receptor (GLP-1R) agonists, such as exendin-4, are currently approved for the treatment of T2DM. As obesity and T2DM are associated with increased risk of breast cancer, we aimed to explore the effects of GLP-1 and exendin-4, on breast cancer cells. Treatment with GLP-1 or exendin-4 reduced viability and enhanced apoptosis of breast cancer cells but did not affect viability of nontumorigenic cells. Moreover, exendin-4 attenuated tumor formation by breast cancer cells in athymic mice. Treatment with either GLP-1 or exendin-4 elevated cAMP levels, activated the down-stream target CREB, and enhanced CRE promoter transcription, in breast cancer cells. Moreover, inhibition of exendin-4-induced adenylate cyclase activation restored cell viability, thus suggesting cAMP as a principle mediator of exendin-4 anti-tumorigenic activity. While the pancreatic form of the GLP-1R could not be detected in breast cancer cells, several lines of evidence indicated the existence of an alternative GLP-1R in mammary cells. Thus, internalization of GLP-1 into MCF-7 cells was evidenced, infection of MCF-7 cells with the pancreatic receptor enhanced proliferation, and treatment with exendin-(9–39), a GLP-1R antagonist, further increased cAMP levels. Our studies indicate the incretin hormone GLP-1 as a potent inducer of cAMP and an inhibitor of breast cancer cell proliferation. Reduced GLP-1 levels may, therefore, serve as a novel link between obesity, diabetes mellitus, and breast cancer.

Keywords Breast cancer · Glucagon-like peptide-1 · Exendin-4 · cAMP · Type 2 diabetes

Introduction

Breast cancer is the most common malignant tumor among women, affecting up to one in eight women in Western countries [1]. Obesity and type 2 diabetes mellitus (T2DM) are associated with increased risk of breast cancer, and up to 16% patients with breast cancer who are older than 65 years also have T2DM [2]. Several factors may associate obesity and diabetes with breast cancer risk, and include activation of the insulin and insulin-like growth factor 1 (IGF-1) pathways, altered regulation of endogenous sex-hormones, and altered levels of adipocytokines [2–6].

The incretin system is a network of hormones secreted from the gastrointestinal tract in response to food ingestion and may be responsible for up to 70% of postprandial
insulin secretion [7]. Glucagon-like peptide (GLP-1) belongs to the incretin system and is secreted from intestinal L-cells. It exerts its actions through the GLP-1 receptor (GLP-1R)—a G protein-coupled receptor (GPCR) expressed on pancreatic β cells and other tissues, including the brain, heart, and smooth muscle [7]. Several activities of GLP-1 have been described to date. GLP-1 induces insulin secretion from β cells, promotes their proliferation and differentiation, and inhibits their apoptosis [8]. GLP-1 is also a potent inhibitor of gastric emptying and a promoter of satiety; sustained activation of the GLP-1R is associated with weight loss [9]. Indirect evidence suggests that GLP-1 can increase insulin sensitivity in peripheral tissues [10, 11]. These activities indicated GLP-1 as a potential novel therapy for T2DM. As circulating GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP4) and its half-life is 1–2 min, more stable GLP-1R agonist compounds were sought. Exendin-4 is a potent GLP-1R agonist, which is not prone to degradation by DPP4 and its half-life is in the range of 4–8 h [12, 13]. Treatment of diabetic patients with exendin-4 improves glycemic control, reduces insulin resistance, and is associated with weight loss; a synthetic exendin-4 (generic name exenatide) has been approved by the FDA for the treatment of T2DM [14, 15]. Additional GLP-1 analogues and DPP4 inhibitors have also been approved recently for this indication. As GLP-1 enhances proliferation of pancreatic β-cells, the ability of its analogues to promote tumorigenesis has been of concern. While exendin-4 did not enhance proliferation of pancreatic adenocarcinoma cells [16], a recent analysis of the FDA database suggested increased risk of pancreas and thyroid cancers, but not of other cancer types, after treatment with exendin-4 [17].

As T2DM, obesity, and breast cancer commonly occur together [2], any effect GLP-1-based therapy has on breast cancer may have important clinical implications. We aimed to evaluate the effects of GLP-1 and exendin-4 on breast cancer cell proliferation and viability, and show in this study, for the first time, that GLP-1 and exendin-4 inhibit in vitro and in vivo growth of breast cancer cells, while not affecting the growth of non-tumorous mammary cells or primary liver cells. GLP-1 and exendin-4 effects are mediated by cAMP production, and indirect evidence suggest that an alternative GLP-1 receptor is involved in this process.

**Materials and methods**

**Chemicals, antibodies, and constructs**

Exendin-4, GLP-1, exendin-(9–39), 2’,5’-dideoxyadenosine (ddA), and forskolin were obtained from Sigma (St. Louis, MO). SB203580 was obtained from Calbiochem (Gibbstown, NJ). Antibodies used in this study: anti-p53, -PARP, -p21, and P38 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-AKT1 (S473) and total pan-AKT (Cell Signaling Technology, Danvers; MA), anti-diphosphorylated -total ERK 1/2 (Sigma, St. Louis, MO); and anti-phospho-p38 (R&D, McKinley Place NE, MN).

**Cells**

MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells; HEK-293; and rat insulinoma RIN 1046-38 cells were obtained from the American Type Culture Collection (Manassas, VA). HB2, non-tumorigenic mammary epithelial cell line was developed by Dr. J. Taylor-Papadimitriou [18]. Adult human primary liver cells were obtained, after institutional review board’s approval, from clinical liver specimens and cultured as described [19].

**Viral infection**

MCF-7 cells were infected with recombinant adenoviruses at 10 moi (multiplicity of infection) for 3 days. Adenoviruses used in this study: Ad-CMV-GLP-1R, a generous gift of D. Drucker (University of Toronto), and Ad-CMV-GFP (Clontech, BD Biosciences, Mountain View, CA).

**Colony assays**

200–300 cells/well were plated on 12-well plates. 24 h later, medium was replaced, and cells were incubated with either exendin-4 or a control vehicle. Medium was replaced every 2 days, and at day 14, the cells were fixed and stained using 0.5% crystal violet (Sigma). For GLP-1 colony assay, cells were seeded as above, but GLP-1 was added daily in serum-free medium for 2 h, and then serum-containing medium was added.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay

2–3×10^3 cells/well were plated in 96-well plates, cultured in the appropriate media, and viability was evaluated using MTT as described [20].

**Apoptosis analysis**

7×10^3 cells were plated in the appropriate culture media containing 10% FCS. Cells were then serum starved for 24 h, and treated with either control vehicle or exendin-4. After 48 h, cells and medium were collected and stained with PI and Annexin V, using the Annexin V-PE Apoptosis Detection Kit I (MBL, Woburn, MA) according to the
manufacturer protocol. Flow cytometry was performed using FACScan (Becton–Dickinson, Franklin Lakes, NJ).

Western blot analysis

Cells were harvested, lysed and total protein was extracted with RIPA buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM NaF) together with a protease inhibitor cocktail (Sigma). Lysates were resolved on 10% SDS-PAGE and immunoblotted with the indicated antibodies. Band intensities were quantified using ImageJ software.

cAMP measurements

Cells were seeded in 24-well plates, and a day later treated as indicated. Plates were snap-frozen in liquid nitrogen and stored at −70°C until further analyses. Samples were diluted in 50 mM sodium acetate pH 6.2, acetylated, and incubated overnight in 4°C with [125I]-cyclic AMP (Perkin Elmer, Waltham, MA) and anti-cAMP antibody (Sigma, no. A0670). The following day 1% BSA and ethanol were added, samples centrifuged, and cAMP was measured in precipitate using an automatic gamma counter (Perkin Elmer).

cAMP response element–luciferase assay

MCF-7 cells were plated in 24-well plates, at a confluency of ~60%, transfected using LipofectAMINE 2000 (Invitrogen) with the p-cAMP responsive element (pCRE)–luciferase (pCRE–LUC) reporter plasmid (Clontech) and serum-starved for 24 h, then treated as indicated. Luciferase activity was evaluated by Luciferase Assay System (Promega, Madison, WI).

Animal studies

Experiments were performed under institutional guidelines established for the Animal Core Facility at the Sheba Medical Center. Six-week-old female athymic nude mice were injected subcutaneously in both flanks with 0.5 × 10^6 MDA-MB-231 cells suspended in 100 µl PBS/matriigel (BD Biosciences, San Jose, CA). Five days later, the mice were implanted subcutaneously with 28-day osmotic pumps (Alzet, Cupertino, CA) delivering continuously vehicle (PBS) or exendin-4 (500 ng or 2 µg per day). Tumor size was measured with a linear digital caliper weekly. Volume was estimated using the equation V = (a × b^2) × 0.5236, where “a” is the larger dimension, and “b” the perpendicular diameter [21]. A similar experiment was conducted using MDA-MB-468 cells (1.8 × 10^6 cells/flank) and intra-peritoneal injection of PBS or exendin-4 (5 days/week; 500 ng or 2 µg per day).

GLP-1 internalization assay

5 × 10^4 RIN 1046-38 cells, expressing classical GLP-1R [23] or MCF-7 cells, were seeded on 13-mm round cover slip glass in 24 well plates. On the next day, cells were incubated with 1 µM rhodamine-labeled GLP-1(7–36)-amide (Phoenix Pharmaceuticals, Burlingame, CA) in serum-free medium and fixed after 0, 1, 5, 10, 30, and 60 min with cold 4% paraformaldehyde and washed three times with 100 mM glycine. Internalization was monitored using Leica TCS SP5 confocal microscopy system.

Statistical analysis

The data are expressed as mean ± SD. Study variables were compared between the study groups using Fisher’s exact test for categorical variables and Student’s t test for continuous variables. All the calculations were two tailed. P values of <0.05 were considered statistically significant.

Results

GLP-1 and exendin-4 inhibit growth of breast cancer cells

The effect of GLP-1 on growth of MCF-7 (estrogen-receptor (ER)-positive) and MDA-MB-231 (ER-negative) breast cancer cells was assessed by colony formation assay.
GLP-1 is rapidly degraded by the protease DPP4 [24]. MCF-7 and MDA-MB-231 do not express DPP4 (data not shown), but it might be present in the serum. The cells were, therefore, treated daily with GLP-1 in serum-free media for 2 h and then serum-containing medium was added. GLP-1 treatment (50 nM) significantly decreased the number of colonies (Fig. 1a). Next, the effect of exendin-4 on colony formation was assessed. Cells were treated with exendin-4 (1, 10, 50 nM) or control vehicle for 14 days. Exendin-4 significantly reduced the number of colonies formed by MCF-7 cells and MDA-MB-231 cells, but did not affect colony formation of HB2 (non-cancerous HB2 mammary cells) or human primary liver cells (Fig. 1b). As GLP-1 and exendin-4 showed similar activities, but exendin-4 is more stable, the following experiments were conducted using exendin-4.

Short-term effects of exendin-4 on breast cancer cell growth were determined by MTT assay. MCF-7, MDA-MB-231, MDA-MB-468, HB2, and HEK-293 cells and human primary liver cells were treated with exendin-4 as indicated. Exendin-4 did not affect the growth of non-cancerous cells, but up to 50% growth inhibition of breast cancer cells was observed, with maximal effect at 0.5–10 nM exendin-4 concentrations (Fig. 1c).

In accordance with the viability studies, exendin-4 treatment elevated expression of the cell cycle inhibitors p53 and p21 in MCF-7 cells, but reduced their levels in primary liver cells (Fig. 2a). Exendin-4 did not affect p53 and p21 levels in the p53-mutated MDA-MB-231 cells [25]. Cyclin D1, a positive regulator of cell cycle, was down-regulated in MCF-7 cells (Fig. 2b). Exendin-4 treatment increased apoptosis in MCF-7 cells (Fig. 2c, d) and MDA-MB-231 cells (data not shown), as evidenced by annexin-V staining (5% of apoptotic cells in untreated versus 19% in treated cells, P < 0.01), and increase PARP cleavage (Fig. 2d).

Exendin-4 sensitizes MCF-7 cells to chemotherapy

Exendin-4 increased sensitivity of MCF-7 cells to doxorubicine (Fig. 3a) or paclitaxel (Fig. 3b), as evidenced by MTT assays. Thus, 0.5 μM doxorubicine reduced viability to 33%, and with exendin-4 it decreased to 8%; 0.5 μM paclitaxel reduced the viability to 18%, while the addition of exendin-4 reduced it to 3%.

Exendin-4 inhibits growth of breast cancer cells in vivo

The ability of exendin-4 to slow tumor formation was studied using two cell lines. MDA-MB-468 cells were injected into both flanks of nude mice (5 mice per group, 2 tumors per mouse), and 12 days later, mice were treated daily with intraperitoneal injection of exendin-4 (500 ng or 2 μg per day), or vehicle (PBS) for 6 weeks. Tumor growth and mice weight were monitored weekly, and harvested tumors were weighed at the end of the experiment. After 3 weeks of exendin-4 treatment a dose-dependent inhibition of tumor growth was seen (P < 0.005 for 2 μg/day, Fig. 4a, upper panel). Tumor weights were lower in exendin-4-treated groups (by 25% for 500 ng and 75% for 2 μg/day, P < 0.001 for 2 μg/day compared to control; Fig. 4a, lower panel).

The in vivo activity of exendin-4 against MDA-MB-231 cells was studied by continuous administration using osmotic pumps containing either vehicle or exendin-4 (500 ng, or 2 μg per day). Cells were injected into both flanks of athymic mice (5 mice in a group), and 5 days later the osmotic pumps were implanted. A dose-dependent inhibition of tumor size was observed (P < 0.05 for 2 μg/day, Fig. 4b). No differences in general health or weight were observed between PBS or exendin-4 treated groups (Fig. 4c).

Exendin-4 increases cAMP levels and activity in breast cancer cells

The effects of GLP-1 and exendin-4 on cAMP levels in MCF-7 cells were examined by radioimmunoassay (RIA). Cells were treated with exendin-4 for 5 min, and maximal effect of 2.5-fold increase was achieved at a concentration of 5 nM (Fig. 5a). Similar effect was noted following treatment with GLP-1 (Fig. 5b). Forskolin, an adenylyl cyclase activator, served as a positive control, and the adenylate cyclase inhibitor ddA, inhibited the upregulation of cAMP by either exendin-4 or GLP-1 (Fig. 5b), indicating adenylate cyclase as the mediator of cAMP upregulation by GLP-1. The transcription factor cAMP response element binding protein (CREB) is downstream to cAMP, and is activated by phosphorylation [26]. Exendin-4 induced CREB phosphorylation in breast cells in a dose-dependent manner (Fig. 5c).

Exendin-(9–39) is an antagonist of the classic GLP-1R in pancreatic cells [24]. However, in various GLP-1R-negative cells, including hepatocytes and adipocytes, exendin-(9–39) shows similar activity to the GLP-1R agonist exendin-4 [27–29]. In MCF-7 cells, exendin-(9–39) enhanced cAMP production and CREB phosphorylation (Fig. 5a, d). Combination of exendin-(9–39) with either exendin-4 or GLP-1 further elevated cAMP production and CREB phosphorylation (Fig. 5a, d).

Upon activation, CREB binds to cAMP response element (CRE) sites, thus inducing transcription of target genes [26]. In accordance with their effects on cAMP levels and CREB phosphorylation, GLP-1 and exendin-4 induced a 2- and 1.7-fold activation of CRE-luciferase (respectively; P < 0.01), and combined treatment with exendin-(9–39) further induced a 4.5-fold activation (P < 0.005; Fig. 5e).
Inhibition of adenylate cyclase abrogates exendin-4 growth inhibitory effects

We examined the role of cAMP in mediating exendin-4 growth inhibitory effect. MCF-7 cells were treated with increasing exendin-4 concentrations, alone or in combination with ddA. At low exendin-4 concentrations (0.5 and 1 nM), ddA abrogated exendin-4 effects on cell viability (Fig. 5f). Similarly, cAMP is known to inhibit growth of breast cancer cells [30], and while forskolin reduced cell viability, ddA abolished the effect of low doses of forskolin (0.05–1 μM) (Fig. 5g).
Exendin-4 induces p38 activation

We studied the effects of exendin-4 on stress-related pathways. cAMP and its analogues, including forskolin, were found to activate p38 MAPK [31]. Indeed, we observed p38 MAPK phosphorylation in breast cancer cell lines upon forskolin and exendin-4 treatment (at concentrations as low as 1 nM; Fig. 6a). In pancreatic β-cells, GLP-1 and exendin-4 activate a subset of pathways, including AKT and ERK1/2 [32, 33]. However, exendin-4 (1–100 nM, 15 min) did not activate ERK1/2 in MCF-7 cells (Fig. 6a). Moreover, a slight but consistent decrease in AKT phosphorylation was observed following exendin-4 treatment (Fig. 6a, b).

cAMP-mediated activation of p38 has been shown as a mechanism leading to cancer cell death [31]. Therefore, we studied exendin-4-mediated cell death following p38

Fig. 2 Exendin-4 induces cell cycle arrest and apoptosis in breast cancer cells. a MCF-7, MDA-MB-231 cell lines, and primary liver cells were treated by exendin-4 (5 nM). After 48 h, cells were harvested, separated on SDS-PAGE gels, and immunoblotted using anti-p53 or anti-p21 antibodies. β-actin served as loading control. b MCF-7 cells were treated with exendin-4 (5 nM) for the indicated times, cells were harvested, and immunoblotted with anti-cyclin D1 antibodies. c MCF-7 cells were treated with exendin-4 (1 nM) for 48 h. Following treatment, cells were harvested and stained for PI and annexin V, as described under “Materials and Methods” section. Representative results are shown in the upper panel. Results of three independent experiments are shown in bars. *P < 0.05, **P < 0.01. d MCF-7 cells were treated with 5 nM exendin-4 for the indicated times, harvested and analyzed by Western blotting for the expression of PARP. Arrows indicate full length and cleaved PARP.

Fig. 3 Exendin-4 increases MCF-7 cell sensitivity to chemotherapy. MCF-7 cells were treated with elevated doxorubicine (a) or paclitaxel (b) concentrations, with or without exendin-4 (10 nM). MTT assay was conducted at 48 h. *P < 0.01; **P < 0.001, drug alone versus drug with exendin-4. NS not significant.
inhibition. Pre-treatment of MCF-7 cells with the p38 inhibitor SB203580 abrogated exendin-4 inhibitory effect at concentrations of as low as 0.5 nM exendin-4. The effect was diminished at higher exendin-4 concentrations (50 and 100 nM; Fig. 6c).

Evidence for an alternative GLP-1 receptor in breast cancer cells

The activities of exendin-4 in pancreatic cells are mediated through the GLP-1R [7, 9]. We attempted to detect GLP-1R in cancerous and non-cancerous breast cells. The receptor was easily detected by conventional PCR or RT-PCR in human pancreatic islet samples (Fig. 7a, lane 1, and data not shown), but was not detected by either method in six mammary cell lines (Fig. 7a, lanes 2, and data not shown), despite using multiple primer sets targeting different regions of the GLP-1R-coding sequence.

Internalization of a ligand may serve as indirect evidence to the existence of a receptor. As GLP-1R could not be detected, we studied GLP-1 internalization into breast cancer cells. RIN 1046-38, a rat insulinoma cell line which...
Fig. 5  
cAMP pathway mediates exendin-4 and GLP-1 activities in MCF-7 cells.  

**a** MCF-7 cells were treated for 5 min with exendin-4, at indicated concentrations, and cAMP levels measured. **P < 0.005** for exendin-4 treatments compared to control; *P < 0.02 for 100 and 500 nM vs. 5 nM exendin-4. 

**b** MCF-7 cells were treated for 5 min with exendin-4 (5 nM; Ex4), GLP-1 (5 nM), exendin-(9–39) (100 nM; Ex9) or forskolin (1 µM), and in combination of exendin-4 or GLP-1 with exendin-(9–39) or 2′,5′-dideoxy-adenosine (ddA, 30 µM), and cAMP levels were measured. Results are shown relative to vehicle-treated cells. **P < 0.05 for all treatments compared to control, except for exendin-4 + exendin-(9–39), *P < 0.01. 

**c** Cells were treated with increasing concentrations of exendin-4, or forskolin, for 15 min, harvested and analyzed by Western blotting for the expression of pCREB. 

**d** Cells were treated for 15 min with exendin-4, exendin-(9–39), combination of the two compounds or forskolin, harvested and analyzed by Western blotting for the expression of pCREB. 

**e** Transcription of CRE-promoter was evaluated in MCF-7 cells transfected with CRE-luciferase reporter plasmid. Cells were treated for 6 hrs with exendin-4 (50 nM), GLP-1 (50 nM), exendin-(9–39) (50 nM), and in combination of exendin-4 or GLP-1 with exendin-(9–39). Luciferase activation was measured as described under  “Materials and Methods” section. Experiments were conducted in quadruplicates and results are shown as fold increase of vehicle treated cells. *P < 0.01, **P < 0.005. 

**f** Adenylate cyclase inhibition abrogates exendin-4-mediated cell death. MCF-7 cells were seeded in 96-well plates and treated with increasing exendin-4 (f) and forskolin (g) concentrations, or in combination with 2′,5′-dideoxyadenosine (ddA). Viability was determined with MTT assay after 48 h. ***P < 0.001; **P < 0.005; *P < 0.05, with ddA versus without ddA.
expresses GLP-1R [23], and MCF-7 cells were incubated with rhodamine-labeled GLP-1 (7–36)-amide (1 μM) for 0, 1, 5, 10, 30 and 60 min. After 5-min, dense red labeling, reflecting internalized GLP-1, was observed in the cytoplasm of both cell lines (Fig. 7b, c), in a granule-like appearance. Labeling declined after 10 min, and almost disappeared at 60 min (data not shown).

The absence of GLP-1R expression and agonistic effects of the GLP-1R antagonist exendin-(9–39) in breast cancer cells prompt us to study the effects of the pancreatic

![Image](image_url)
Fig. 7 An alternative GLP-1R is active in MCF-7 cells. a GLP-1R expression in MCF-7 breast cancer cell line. RT-PCR was performed using 2 μg total RNA isolated from human pancreas and from MCF-7 cell line either not infected, or infected with adeno-GFP or adeno-GLP-1R. PCR products were analyzed by agarose gel electrophoresis (β actin control; bottom panel). b Internalization of GLP-1 into RIN 1046-38 and MCF-7 cells. Cells were incubated for 5 min with rhodamine-labeled GLP-1 (7–36)-amide and fixed. Representative pictures for each cell line showing internalization of labeled GLP-1 yielding dense red granule-like fluorescence within the cells cytoplasm. c Columns represent average fluorescence intensity of at least three fields of three independent experiments as performed in b (***P < 0.005). d, e, f GLP-1R increases MCF-7 cell proliferation and activates GLP-1R-related signaling pathways. d MCF-7 cells were infected with adeno-GLP-1R or control adeno-GFP, and 24 h later 200 cells of each treatment, were incubated as indicated with 5 nM exendin-4. Two weeks later, cells were fixed, stained, and quantified using ImageJ. *P < 0.05, adeno-GFP exendin-4 versus control. **P < 0.01, adeno-GLP-1R versus adeno-GFP. e Cells were infected as in d, and 4 days later infected cells were seeded, and treated in quadreplicates as indicated with GLP-1, exendin-4 (each 5 nM), exendin 9–39 (100 nM), or control for 5 min. Cells were snap-frozen, and cAMP levels were measured using RIA. Exendin 9–39 inhibited exendin-4-induced cAMP production in GLP-1R-expressing cells only. *P < 0.005, for GLP-1 or exendin-4-treated GLP-1R-infected cells versus same treatment with addition of exendin 9–39. f MCF-7 cells were infected as in d, treated for 15 min with exendin-4, lysed and immunoblotted as indicated.
GLP-1R in breast cancer cells. To this aim, we conducted a series of colony assays following infection of MCF-7 cells with either GLP-1R-expressing (MCF-7/GLP-1R) or GFP-expressing adenovirus. Cell number and colonies of MCF-7/GLP-1R cells were increased compared to GFP-infected cells (Fig. 7d). Importantly, while exendin-4 reduced colony number of the control cells, it did not inhibit colony formation of MCF-7/GLP-1R cells.

We examined possible signaling pathways acquired by MCF-7/GLP-1R following GLP-1R expression. Treatment of MCF-7/GLP-1R cells with exendin-4 enhanced cAMP production and increased phosphorylation of ERK1/2 and AKT (Fig. 7f). Similar to its role in GLP-1R expressing pancreatic cells, exendin (9–39) inhibited the cAMP production in MCF-7/GLP-1R cells (Fig. 7e).

**Discussion**

We report in this article the growth inhibitory effects of GLP-1 and exendin-4 on breast cancer cells. The effect was specific to breast cancer cells, was not observed in non-malignant mammary epithelial cells or primary liver cells, and was associated with production of cAMP. While the classic GLP-1R could not be detected in breast cancer cells, our data suggest the existence of an alternative receptor in mammary cells.

Breast cancer and T2DM have been associated with increased breast cancer risk and with adverse prognosis among breast cancer patients [2–6]. The mechanisms involved in this interaction are not fully elucidated and may include increased serum insulin levels, increased activation of the IGF-1 pathway, increased production of sex hormones by the adipose tissues, and altered regulation of adipokines [34, 35]. However, additional factors may also be involved. T2DM is associated with reduced postprandial secretion and activity of GLP-1 [36–39]. Thus, in light of our results, reduced GLP-1 levels in diabetes may serve as an additional mechanism linking between diabetes and increased breast cancer risk.

Despite extensive search, we did not detect the classic GLP-1R in breast cancer and non-tumorigenic mammary cells. Our studies suggest the existence of an alternative receptor in these cells. We observed internalization of GLP-1 into MCF-7 cells, in a similar kinetics and appearance observed in GLP-1R expressing cells (Fig. 7b, c and [40, 41]). Infection of MCF-7 cells with the pancreatic GLP-1R increased their proliferation and enhanced activation of growth-promoting signaling pathways. Finally, the GLP-1R antagonist exendin-(9–39) acted as an agonist and increased cAMP levels in breast cancer cells. Ample data support the existence of an alternative GLP-1 receptor [42]. For example, GLP-1, as well as exendin-(9–39), induces glycogenic effect in hepatocytes and muscle cells, which do not express the classic GLP-1R and, similar to our observation, forced expression of GLP-1R reversed this effect [28, 29]. Binding assays detected a 63 KDa protein, similar to GLP-1R mass in these cells [28]. GLP-1R is not expressed in fat tissue, but treatment with GLP-1 elicited insulinomimetic effects in 3T3-L1 cells, and exendin-(9–39) enhanced the effect [27]. GLP-1(9–36) amide, a GLP-1 cleaved product, directly suppressed glucose production in isolated mouse hepatocytes ex vivo independent of the GLP-1R, an effect augmented by exendin-(9–39) [43]. Finally, we have recently shown that treatment of human primary liver cultures (negative to GLP-1R) with exendin-4 increased cell proliferation and enhanced PDX-1-induced liver to pancreas trans-differentiation process [44].

The effect of GLP-1 in β-cells is mediated by a GPCR, which leads to cAMP elevation, activation of protein kinase C, MAPK ERK1/2, AKT, and CREB [45]. cAMP and its analogue 8-Cl-cAMP induce apoptosis and growth arrest in cancer cells, elevate levels of p21 and p27, and inhibit growth of breast, prostate, ovary, and colon cancers [30, 46, 47]. GLP-1 and exendin-4 elevate cAMP in breast cancer cells (Fig. 5a, b) and exendin-4 growth-inhibitory activity was reversed by impairment of adenylate cyclase activity (Fig. 5f), thus suggesting that upregulation of cAMP mediates exendin-4 effects on breast cancer cell growth.

A potential target for cAMP-related growth inhibition is p38 MAPK, which is usually related to growth inhibition or apoptosis [48]. p38 MAPK activity can be regulated by cAMP: for example, cAMP can induce p38 activation in NG108-15 neuroblastoma glioma hybrid cells [49] and SK-N-MC human neuroblastoma cells [50]. We observed activation of p38 following GLP-1 treatment (Fig. 6a) and found that p38 inhibition abolished exendin-4–anticancer effects, suggesting a link between GLP-1, cAMP, and p38.

The anti-proliferative effect of exendin-4 was detected at 0.5–10 nM, and was diminished at 50–100 nM (Figs. 1c, 5f, 6c). Similarly, cAMP accumulation peaked at 50 nM, and decreased at 100 and 500 nM (Fig 5a). A possible explanation to this observation is saturation and desensitization of the receptor upon exposure to high concentrations of the ligand. In addition to activating cAMP, GLP-1 has also been shown to activate the β-arrestin pathway, which can lead to inactivation of the pro-apoptotic protein Bad and inhibit apoptosis [51]. It is possible that activation of the β-arrestin pathway, which occurs only upon sustained stimulation of the receptor, may be involved in the reduced growth inhibition observed at higher concentrations of GLP-1.

Given the increasing incidence of T2DM, more women are expected to be exposed to GLP-1R agonists. Yet, the
effects of this class of medications on malignant diseases have not been examined thoroughly, and unpublished data are limited by sample size and time of follow-up (www.fda.gov). Our study suggests that GLP-1R agonists may not be associated with increased breast cancer risk. Similar to our data, exenend-4 did not induce cell proliferation and even seemed to slow growth of pancreatic cancer cells in nude mice [16]. There are ongoing efforts to develop longer-acting GLP-1R agonists with extended circulating pharmacokinetic profiles. Our results, showing reduced tumor growth upon continuous exendin-4 administration using osmotic pumps, suggest that long-acting GLP-1R agonists may be safe.

In conclusion, our studies indicate the incretin hormone GLP-1 as a potent inducer of cAMP and an inhibitor of breast cancer cell proliferation. Reduced GLP-1 levels may, therefore, serve as a novel link between obesity, diabetes mellitus, and breast cancer.

Acknowledgments This research was supported by The Riva and Joel Koschitzky Fund for Breast Cancer Research at the Sheba Medical Center; the “Talpiut” Sheba Career Development Award. We thank Mrs. Iris Raviv to her technical assistance.

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