CI-988 inhibits EGFR transactivation and proliferation caused by addition of CCK/Gastrin to lung cancer cells

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Abstract

Cholecystokinin (CCK) receptors are G-protein coupled receptors (GPCR) which are present on lung cancer cells. CCK-8 stimulates the proliferation of lung cancer cells, whereas the CCK2R receptor antagonist CI-988 inhibits proliferation. GPCR for some gastrointestinal hormones/neurotransmitters mediate lung cancer growth by causing epidermal growth factor (EGF)R transactivation. Here, the role of CCK/gastrin and CI-988 on EGFR transactivation and lung cancer proliferation were investigated. Addition of CCK-8 or gastrin-17 (100 nM) to NCI-H727 human lung cancer cells increased EGFR Tyr^{1068} phosphorylation after 2 min. The ability of CCK-8 to cause EGFR tyrosine phosphorylation was blocked by CI-988, gefitinib (EGFR tyrosine kinase inhibitor), PP2 (Src inhibitor), GM6001 (matrix metalloprotease inhibitor) and tiron (superoxide scavenger). CCK-8 nonsulfated and gastrin-17 caused EGFR transactivation and bound with high affinity to NCI-H727 cells, suggesting that the CCK2R is present. CI-988 inhibited the ability of CCK-8 to cause ERK phosphorylation and elevate cytosolic Ca^{2+}. CI-988 or gefitinib inhibited the basal growth of NCI-H727 cells or that stimulated by CCK-8. The results indicate that CCK/gastrin may increase lung cancer proliferation in an EGFR-dependent manner.

Keywords

CCK; gastrin; CI-988; EGFR transactivation; lung cancer proliferation

Introduction

Cholecystokinin (CCK), an 8 amino acid peptide, is biologically active in the central nervous system and periphery (Mutt and Jorpes, 1971). In the CNS, CCK alters rodent behavior, neuronal activity and causes satiety (Gibbs et al., 1973, Crawley, 1988; Wang 1986). In the periphery, CCK is localized to the myenteric plexus and endocrine cells. Upon secretion, CCK causes release of enzymes from the pancreas and contraction of the gallbladder (Jensen et al., 1980). In the stomach, gastrin-17 causes release of gastric acid

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which facilitates digestion of fats and proteins. The actions of CCK-8/gastrin are mediated by two receptors (R), the CCK1R and CCK2R (Dufresne et al., 2006). The CCK1R binds CCK-8 sulfated but not nonsulfated (NS) CCK-8 with high affinity and is antagonized by L-364,718 (Jensen et al., 1989). The CCK2R binds gastrin-17, CCK-8 and CCK-8NS with high affinity and is antagonized by L-365,260 (Innes and Snyder, 1980). In the normal CNS, pancreas and stomach, both the CCK1R and CCK2R are present in many species (Dufresne el al., 2006).

The signal transduction mechanisms of the CCK2R have been extensively investigated. The CCK2R is a 447 amino acid G-protein coupled receptor (GPCR) which causes phosphatidyl inositol (PI) turnover (Wank et al., 1998). The diacyglycerol released activates protein kinase (PK) C, whereas the inositol-1,4,5-trisphosphate released causes increased cytosolic Ca^{2+} (Staley et al., 1989; Sethi and Rozengurt, 1991). Other downstream targets include ERK, FAK, Jun kinase, p38 MAP kinase and PI-3-K (Dockray et al., 2012). CCK-8 stimulates whereas L-365,260 and CI-988 inhibit SCLC basal growth or that stimulated by CCK-8 (Moody and Jensen, 2001). Several cancers make gastrin which functions as an autocrine growth factor (Rehfeld, 2006). The CCK2R is present in many cancers, including small cell lung cancer (SCLC), medullary thyroid carcinomas, astrocytomas and stromal ovarian cancers (Reubi et al., 1997).

SCLC is a neuroendocrine tumor with p53 mutations which kills approximately 30,000 patients is the United States (USA) annually. SCLC patients are usually treated with chemotherapy. In contrast, non-SCLC (NSCLC) is an epithelial tumor with epidermal growth factor receptor (EGFR) amplifications and mutations, which kills approximately 130,000 USA patients annually (Kaufman et al., 2011). In the world, lung cancer kills approximately 1,500,000 patients annually. NSCLC patients who fail chemotherapy can be treated with the tyrosine kinase inhibitors (TKI) erlotinib and gefitinib (Lynch et al., 2004; Paez et al., 2004). The EGFR is activated by endogenous ligands such as transforming growth factor (TGF) α resulting in tyrosine phosphorylation of the EGFR, ERK and PI-3-K (Lemmon and Schlessinger, 2010). Also, GPCR can cause EGFR tyrosine phosphorylation through transactivation (George et al. 2013). Previously, we found that GPCR for bombesin, neurotensin or pituitary adenylate cyclase activating polypeptide caused EGFR transactivation in NSCLC cells (Moody et al., 2010, Moody et al., 2012; Moody et al., 2014). In this communication, CCK-8 causes lung cancer EGFR transactivation and proliferation. Further CI-988 antagonized the increase in EGFR transactivation and proliferation caused by CCK-8 addition to lung cancer cells.

Materials and Methods

Cell culture and receptor binding

NCI-H727 cells were cultured in RPMI-1640 containing 10% fetal bovine serum. The cells were split weekly 1/20 with trypsin-ethylenediaminotetraacetic acid (EDTA). The cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO_2/95% air. For the radioreceptor assay NCI-H727 cells were placed in 24 well plates. When confluent the cells were washed 3 times in SIT medium (RPMI-1640 containing 3×10^{-8} M sodium selenite, 5 μg/ml bovine insulin and 10 μg/ml

transferrin (Sigma-Aldrich, St. Louis, MO)). The cells were incubated in SIT buffer containing 0.25% bovine serum albumin and 250 μg/ml bacitracin (Sigma-Aldrich, St. Louis, MO) and ^{125}I-Bolton Hunter(BH)-CCK-8 (50,000 cpm) added as well as various concentrations of CCK-8, nonsulfated (NS)CCK-8, CCK-4, gastrin-17 or CI-988. After incubation at 37°C for 30 min, free ^{125}I-BH-CCK8 was removed by washing 3 times in buffer and the cells which contained bound ^{125}I-BH-CCK8 dissolved in 0.2 N NaOH and counted in a gamma counter. The Ki was calculated for each unlabeled competitor.

Western Blot

The ability of CCK-8, CCK-8NS, CCK-4 or gastrin-17 (Bachem Inc., Torrence, CA) to stimulate tyrosine phosphorylation of the EGFR or ERK (p42/p44 MAP kinase) was investigated by Western blot. Lung cancer cells were cultured in 10 cm dishes. When a monolayer of cells formed they were placed in SIT media for 3 hr. Routinely, lung cancer cells were treated with CI-988 (Provided by Dr. J. Hughes and dissolved in DMSO at a concentration of 30 mM), gefitinib (Tocris Bioscience, Bristol, UK, and dissolved in DMSO at a concentration of 30 mM), PP2 (Sigma-Aldrich, St. Louis, MO and dissolved in DMSO at a concentration of 10 mM), GM6001 (Sigma-Aldrich, St. Louis, MO and dissolved in DMSO at a concentration of 1 mM), N-acetylcysteine (NAC, Sigma-Aldrich, St. Louis, MO and dissolved in DMSO at a concentration of 5 M), Tiron (Sigma-Aldrich, St. Louis, MO and dissolved in DMSO at a concentration of 5 M) or no additions for 30 minutes. Then cells were incubated with 0.1 μM CCK-8 for 2 min, washed twice with PBS and lysed in buffer containing 50 mM Tris.HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 1% sodium azide, 1 mM ethyleneglycoltetraacetic acid, 0.4 M EDTA, 1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride and 0.2 mM sodium vanadate (Sigma-Aldrich, St. Louis, MO). The lysate was sonicated for 5 s at 4°C and centrifuged at $10,000 \times g$ for 15 min. Protein concentration was measured using the BCA reagent (Pierce Chemical Co., Rockford, IL), and 400 μg of protein was incubated with 4 μg of anti-phosphotyrosine (PY) monoclonal antibody, 4 μg of goat anti-mouse immunoglobulin IgG and 15 μl of immobilized protein G overnight at 4°C. The immunoprecipitates were washed 3 times with phosphate buffered saline and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting. Immunoprecipitates were fractionated using 4–20% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes and the membranes were blocked overnight at 4°C using blotto (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide) and incubated for 16 h at 4°C with 1 μg/ml anti-EGFR antibody (Cell Signaling Technologies, Danvers, MA) followed by anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Upstate Biotechnologies, Lake Placid, NY). The membrane was washed for 10 min with blotto and twice for 10 min with washing solution (50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). The blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to Kodak XAR film. The band intensity was determined using a densitometer.

Alternatively, 20 μg of cellular extract was loaded onto a 15 well 4–20% polyacrylamide gels. After transfer to nitrocellulose, the blot was probed with anti PY[1068]-EGFR, anti-EGFR, anti-PY[204]ERK, anti-ERK or anti-tubulin (Cell Signaling Technologies, Danvers, MA).

Cytosolic Ca^{2+}

NCI-H727 cells were treated with trypsin-EDTA and harvested. After centrifugation the cells were resuspended in SIT medium (2.5×10^6 cells/ml) containing Fura-2AM (Calbiochem, La Jolla, CA) at 37°C for 30 min (Staley et al., 1990). The cells were centrifuged at $1000 \times g$ for 5 min and resuspended at a concentration of 2.5×10^6/ml and 2 ml placed in a Quartz cuvette containing a stirbar. The excitation ratio was determined at 340 and 380 nm and the emission at 510 nm using a spectrofluorometer equipped with a magnetic stirring mechanism and temperature (37°C) regulated cuvette holder before and after addition of drug.

Reactive oxygen species

NCI-H727 cells were placed in black 96 well plates (30,000 cells/well) and cultured overnight. The cells were treated with 10 μM dichlorofluoresceindiacetate for 1 h and washed 3 times with serum- free SIT medium. Some of the cells were treated with 10 μM CI-988 and then stimuli such as 10 nM CCK-8 or 10 μM H_2O_2 added. Fluorescence measurements were taken at the various times using an excitation wavelength of 485 nm and emission wavelength of 585 nm.

Proliferation

Growth studies in vitro were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) and clonogenic assays. In the MTT assay, NCI-H727 cells were placed in SIT medium and various concentrations of CI-988 or gefitinib added. After 2 days, 15 μl of 0.1 % MTT solution added. After 4 h, 150 μl of dimethylsulfoxide was added. After 16 h, the optical density at 570 nm was determined. In the clonogenic assay, the effects of CI-988 and gefitinib were investigated on NCI-H727 cells. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, CCK-8, CI-988 and/or gefitinib using 5×10^4 lung cancer cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet (Sigma-Aldrich, St. Louis, MO) was added and after 16 hours at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μm in diameter were counted using an Omnicon image analysis system.

Results

CCK2R agonists cause EGFR transactivation and bind with high affinity

The ability of CCK/gastrin analogs to cause EGFR transactivation was investigated. Figure 1A shows that addition of 0.1 μM CCK-8, CCK-8NS, gastrin-17 or EGF strongly, whereas CCK-4 moderately increased EGFR tyrosine phosphorylation 2 min after addition to NCI-H727 cells. Fig. 1B shows that CCK-8, CCK-8NS, gastrin-17 and EGF increased

significantly EGFR tyrosine phosphorylation by 233, 149, 208 and 250%, respectively. CCK-4 increase EGFR tyrosine phosphorylation by 125% but it was not significant. In contrast, the ligands had no effect on total EGFR (Fig. 1A). These results indicate that CCK causes EGFR transactivation in lung cancer cells.

Table I shows that CCK-8, CCK-8NS, gastrin-17 and CCK-4 bound with high affinity to NCI-H727 cells with Ki values of 0.8, 1.2, 5.9 and 22 nM, respectively. In contrast, gefitinib, an EGFR tyrosine kinase inhibitor, has no effect. The CCK2R antagonist, CI-988, inhibited specific ^{125}I-BH-CCK-8 binding to NCI-H727 cells with high affinity (Ki = 4.5 nM). These results suggest that the CCK2R is present on NCI-H727 cells. RT-PCR results verified that CCK2R but not CCK1R mRNA was present in NCI-H727 cells (data not shown).

CI-988 is a CCK2R antagonist

The ability of CI-988 to antagonize the effects of CCK/gastrin was investigated. Figure 2A shows that CI-988 inhibited in a dose-dependent manner the ability of CCK-8 to cause EGFR transactivation in NCI-H727 cells. CI-988 at doses of 1 and 10 μM weakly and strongly, respectively, inhibited the ability of 0.1μM CCK-8 to increase EGFR tyrosine phosphorylation. Figure 2B shows that CCK-8 addition to NCI-H727 cells increased EGFR tyrosine phosphorylation by 175% and the increase caused by CCK-8 was significantly inhibited by 10 but not 1 μM CI-988. Similarly, 10 μM but not 1 μM CI-988 antagonized the ability of CCK-8 to cause ERK tyrosine phosphorylation (Fig. 2A). Figure 2C shows that CCK-8 addition to NCI-H727 cells significantly increased ERK tyrosine phosphorylation by 178% and the increase caused by CCK-8 was significantly inhibited by 10 but not 1 μM CI-988. In contrast, CI-988 had no effect on total EGFR or ERK. The results indicate that CI-988 antagonizes the ability of CCK-8 to cause lung cancer EGFR or ERK tyrosine phosphorylation. Similarly, CI-988 antagonized the ability of gastrin-17 to cause EGFR or ERK tyrosine phosphorylation (data not shown).

The effects of CCK/gastrin on cytosolic Ca^{2+} was investigated. Fig. 3 shows that 10 nM CCK-8 or gastrin-17 addition to Fura-2AM loaded NCI-H727 cells increased the cytosolic Ca^{2+} transiently. The cytosolic Ca^{2+} increased rapidly in NCI-H727 cells from 160 to 210 nM within seconds after addition of CCK-8, peaked after about 15 sec and then slowly declined over a 2 min period. L-364,718 had no effect on basal cytosolic Ca^{2+} or the increase caused by CCK-8 (Fig. 3C). CI-988 (1 μM) had no effect on basal cytosolic Ca^{2+} but blocked the increase in cytosolic Ca^{2+} caused by CCK-8 addition to NCI-H727 cells (Fig. 3D). The results demonstrate that CI-988 antagonizes the ability of CCK-8 to cause second messenger production in NCI-H727 cells.

Inhibitors of EGFR transactivation

The cellular mechanism by which CCK-8 causes EGFR transactivation was investigated. Five mM N-acetyl cysteine (NAC) or 5 mM Tiron (Tir) block the ability of CCK-8 to cause EGFR transactivation (Fig. 4A). Figure 4B shows that CCK-8 significantly increased EGFR transactivation by 184% and that the increased in EGFR tyrosine phosphorylation was inhibited significantly by NAC or Tiron. The results indicate that CCK-8 requires reactive

oxygen species (ROS) to cause EGFR transactivation. Table II shows that CCK-8 addition to NCI-H727 cells increases significantly reactive oxygen species (ROS) by 128%. The increase in ROS caused by CCK-8 addition to NCI-727 cells was blocked significantly by CI-988. Fig. 4C shows that CCK-8 addition to NCI-H727 cells increased EGFR tyrosine phosphorylation which was blocked by 10 μM PP2 (Src inhibitor) or 1 μM GM60001 (matrix metalloprotease (MMP) inhibitor). Figure 4D shows that CCK-8 increased the EGFR tyrosine phosphorylation significantly by 214% and the increase was significantly inhibited by PP2 or GM6001. Src may activate MMP causing the metabolism of EGFR precursor ligands to biologically active TGFα (Moody et al., 2010).

CCK-8 stimulates whereas CI-988 or gefitinb inhibit proliferation

The effects of CCK8, CI-988 and/or gefitinib were investigated on NCI-H727 proliferation. Table III shows that addition of 10 nM CCK-8 increases NCI-H727 colony number by 76%. The increase in colony number caused by CCK-8 was reversed by addition of 3 μM CI-988 or 1 μM gefitinib. Surprisingly, CI-988 and gefitinib together reduced NCI-H727 colony number more than either agent alone. In the absence of CCK-8, CI-988 or gefitinib significantly reduced NCI-H727 colony number by 50% and 37% respectively. CI-988 and gefitinib strongly reduced the number of NCI-H727 colonies by 79%. The results indicate that CCK-8 stimulates NCI-H727 proliferation whereas CI-988 or gefitinib inhibit proliferation.

The effects of CI-988 and gefitinib on NCI-H727 proliferation were further investigated. Figure 5 shows that in the absence of CI-988, gefitinib inhibits weakly lung cancer proliferation with an IC_{50} of approximately 30 μM. In the presence of 3 μM CI-988, the gefitinib dose-response curve shifted to the left and the IC_{50} was approximately 5 μM. In the presence of 10 μM CI-988, the IC_{50} for gefitinib was approximately 2 μM. The results indicate that in the presence of CI-988, the cytotoxicity of gefitinib to kill NCI-H727 cells increased by approximately an order of magnitude. The results demonstrate that CI-988 and gefitinib can act synergistically inhibit the proliferation of lung cancer cells.

Discussion

CCK and gastrin as well as the CCK1R and CCK2R are present in certain cancer cells (Clerc et al., 1997; Coulson et al., 2003; Ocejo-Garcia et al., 2001; Sethi et al, 1993). In SCLC cell lines NCI-H209 and NCI-H345, high affinity CCK binding sites were identified (Yoder and Moody, 1987). Both CCK-8 and CCK-8NS bind with high affinity to SCLC cells and increase cytosolic Ca^{2+} (Staley et al., 1989). The effects of CCK-8 on SCLC cells were blocked by L-365–260, the CCK2R antagonist, but not L-364,718, the CCK1R antagonist (Staley et al., 1990). Subsequently CI-988 was developed as a CCK2R antagonist (Hughes et al., 1990). CI-988 inhibits the ability of CCK-8 to increase cytosolic Ca^{2+}, ERK and FAK tyrosine phosphorylation, and c-fos as well as VEGF expression after addition to various SCLC cells (Moody and Jensen 2001). SCLC cells have few EGFR, whereas the EGFR is overexpressed in NSCLC cells which lack the CCK2R (Reubi et al., 1997). Here NCI-H727 cells, a lung carcinoid, were identified as having both EGFR and CCK2R.

The CCK2R activates phospholipase C leading to PI metabolism (Paulson et al., 2000; Jensen et al., 1989). The PI metabolites activate PKC and increase cytosolic Ca^{2+} (Piiper et al., 2003; Staley et al., 1990). High densities of CCK2R mRNA are present on SCLC cell lines NCI-H510, H345 and H69 (Sethi et al., 1993) and addition of 0.1 μM gastrin or CCK-8 to NCI-H510 cells increased cytosolic Ca^{2+} and the increase in cytosolic Ca^{2+} caused by CCK-8 was inhibited by CAM-2200 (CCK2R antagonist). In contrast, addition of gastrin to SCLC GLC19 cells had no effect whereas addition of CCK-8 increased cytosolic Ca^{2+}. The increase in cytosolic Ca^{2+} caused by CCK-8 was inhibited by CAM-1481 (CCK1R antagonist) but not CAM-2200. The results indicate that NCI-H510 has a functional CCK2R whereas GLC19 has a functional CCK1R. Using lung carcinoid cell line NCI-H727, the addition of 10 nM Gastrin or CCK-8 increased the cytosolic Ca^{2+}. The increase in cytosolic Ca^{2+} caused by CCK-8 was inhibited by CI-988 (CCK2R antagonist) but not L364,718 (CCK1R antagonist). Thus the CCK2R but not CCK1R is present on NCI-H727 cells.

Previous studies show that neuropeptides can stimulate cancer cellular proliferation as a result of EGFR transactivation (Moody et al., 2010, 2012, 2014). Addition of CCK-8 to pancreatic cancer cell line AR42J causes tyrosine phosphorylation of numerous proteins. P-Src and P-ERK are significantly increased after CCK-8 addition to AR42J cells (Ferrand et al., 2006). The CCK-8 stimulated increase in P-Src and P-ERK is inhibited by PP2 or GP2A, a Gq inhibitor. Further the increase in EGFR and ERK tyrosine phosphorylation caused by CCK-8 addition to AR42J cells is inhibited by AG1478, an EGFR TKI (Piiper et al., 2003). Gastrin increases heparin binding (HB) EGF, an EGFR ligand, in a PKC-dependent manner (Sinclair et al., 2004). Also, gastrin increases the expression of HB-EGF and amphiregulin which was blocked by the CCK-BR antagonist L-740,093 (Tsutsui et al., 1997). In the present study addition of CCK-8, CCK-8NS or gastrin-17 to NCI-H727 cells significantly increased EGFR tyrosine phosphorylation. The increase in EGFR and ERK tyrosine phosphorylation caused by addition of CCK-8 to NCI-H727 cells was significantly inhibited by CI-988, PP2, gefitinib and GM6001, a MMP inhibitor. In gastric epithelial cells, gastrin increases MMP9 expression (Wroblewski et al., 2002). Preliminary data (T. Moody unpublished) indicate that GM6001 inhibits CCK-8 stimulated secretion of TGF α, an EGFR ligand, from NCI-H727 cells supporting the important role of CCK-8 in MMP activation. Addition of CCK-8 to NCI-H727 cells significantly increased ROS in the present study. The increase in ROS caused by CCK-8 was inhibited by CI-988 and Tiron (T. Moody, unpublished). Also, Tiron inhibited the ability of the CCK2R to transactivate the EGFR. It remains to be determined if CCK increases NAD(P)H oxidase activity resulting in increased ROS production. The results emphasize the importance of MMP-activation, release of EGFR ligands and ROS in the CCK2R regulation of the EGFR.

EGFR transactivation caused by CCK or gastrin addition cells causes numerous downstream events. CCK-8 addition to AR42J cells increases formation of the Shc-Grb2 adaptor protein complex (Piiper et al., 2003). The Src kinase, Yes, is activated by CCK and mediates EGFR Shc-Grb2 interactions (Clerc et al., 2002). Gastrin increases tyrosine phosphorylation of the EGFR as well as ERK and degradation of PPARγ (Chang et al., 2006). Ras, a GTP binding protein, Raf-1, a serine-threonine kinase, and MEK1/2, which phosphorylates ERK, were activated by CCK-8 in a PKC-dependent manner using AR42J cells (Piiper et al., 2003). Transfection of pancreatic cancer cells with a dominant-negative Ras reduces the ability of

CCK to cause EGFR transactivation and ERK tyrosine phosphorylation in AR42J cells (Piiper et al., 2003). MEK1/2, which phosphorylates ERK, is phosphorylated after CCK addition to AR42J cells (Piiper et al., 2003). In the present study, the phosphorylation of ERK caused by CCK-8 is blocked by PD98059, a MEK inhibitor, CI-988 or gefitinib using NCI-H727 cells (T. Moody, unpublished). The results indicate that CCK-8 addition to NCI-H727 cells activates the Ras, Raf, MEK, ERK pathway and that EGFR transactivation is important for this activation.

The activation of ERK by gastrin or CCK leads to a number of important cellular signal cascades. When ERK is phosphorylated it can enter the nucleus and alter gene expression (Whitmarsh and Davies, 1996). Gastrin increases c-fos expression (Stepan et al., 1999) and the c-fos may form a heterodimer with c-jun, activating AP-1 sites. Gastrin addition to intestinal epithelial cells causes increased ELK-1 and COX-2 expression (Guo et al., 2002). Gastrin addition to colon cancer cells increases COX-2 and prostaglandin E2 expression (Colucci et al., 2005). CCK2R activation increases expression of Reg proteins during the early steps of carcinogenesis in the Elas CCK2R mouse pancreas (Gigoux et al, 2008). CCK causes activation of ERK-1 and p85/p110-PI-3-K in a Src- dependent manner (Daulhac et al., 1999). Tyr[438] of the CCK2R is essential for interacting with SHP-2, a protein tyrosine phosphatase, and Akt (Vantinel et al., 2006). Downstream, p70S6-kinase is phosphorylated after addition of CCK-8 (Seva et al., 1997). Activation of the Akt pathway by CCK or gastrin may increase survival of cancer cells.

The EGFR TKI gefitinib or erlotinib are used effectively in NSCLC patients with EGFR mutations who fail chemotherapy. Previously, we showed that non-peptide GRCR antagonists for bombesin, neurotensin and pituitary adenylate cyclase activating polypeptide, increased the potency of gefitinib in NSCLC cells, which have wild type EGFR (Moody et al., 2010, 2012, 2014). Unfortunately, the CCK2R is not abundant in NSCLC cells. In this communication a lung carcinoid cell line NCI-H727, which has wild type EGFR and CCK2R, was identified. We found that CI-988 potentiated the growth inhibitory effects of gefitinib in NCI-H727 cells. Preliminary data indicate that in SCLC cell line NCI-H345, which has wild type EGFR and CCK2R, CI-988 potentiated the growth inhibitory effects of gefitinib (T. Moody, unpublished). Our results suggest that CI-988 can potentiate the effects of gefitinib in some lung carcinoid and SCLC cells suggesting a novel therapeutic approach.

In summary, the CCK2R regulated EGFR transactivation in lung carcinoid cells. CCK-8 or gastrin addition to NCI-H727 cells causes increased tyrosine phosphorylation of the EGFR which is inhibited by CI-988 or gefitinib. CI-988 and gefitinib inhibited synergistically the proliferation of NCI-H727 cells in vitro. The results indicate that some of the growth effects of CCK or gastrin in lung carcinoid cells occur in an EGFR-dependent manner and this could lead to new treatment strategies.

Acknowledgments

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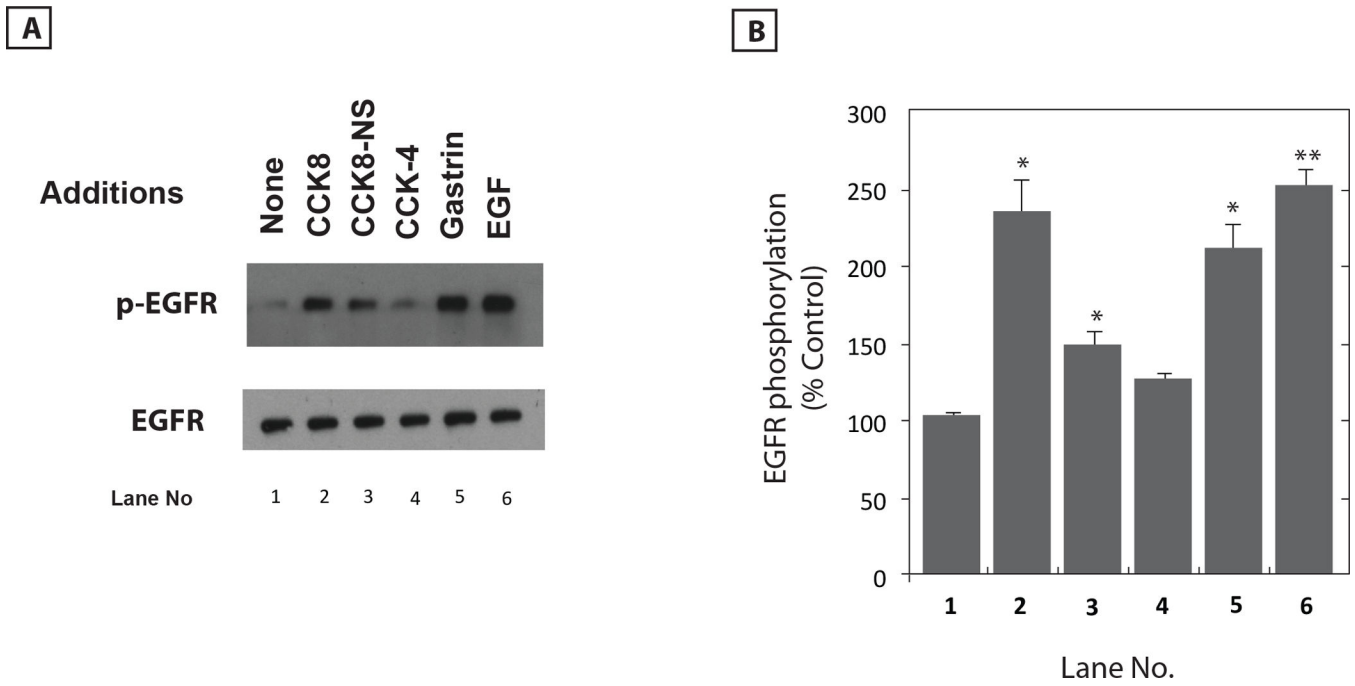
A

B

Fig. 1.
CCK peptides increase EGFR tyrosine phosphorylation. (A) The ability of CCK-8,
CCK-8NS, CCK-4, gastrin-17 or EGF to increase EGFR tyrosine phosphorylation was
investigated using NCI-H727 cells. Total EGFR is indicated. (B) The mean value ± S.E. of 3
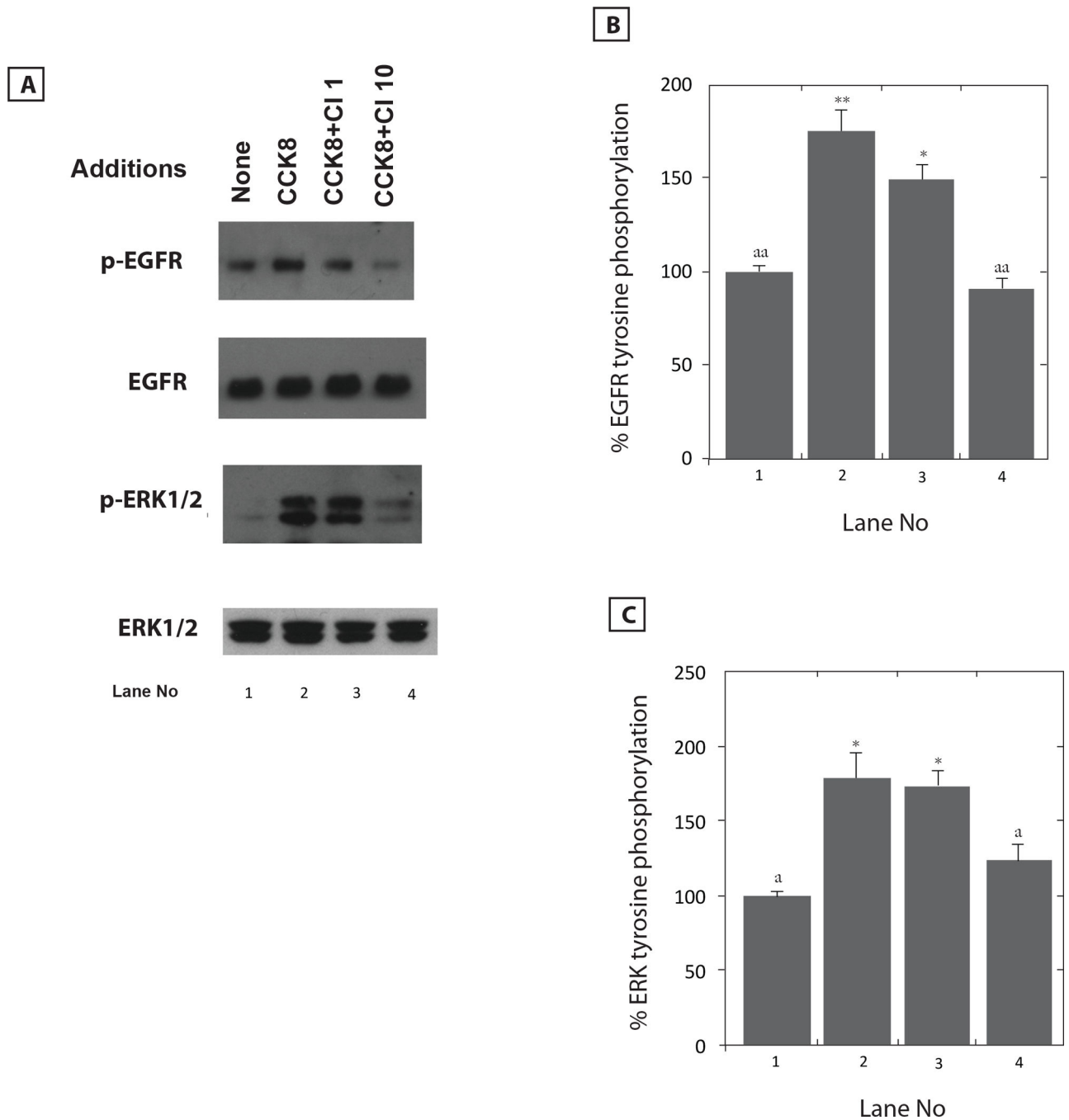experiments is indicated; $p < 0.05$, *; $p < 0.01$, ** relative to control using the Student's t-
test.

Fig. 2.
CI-988 dose-response. (A) Varying doses of CI-988 (CI) were used to antagonize the ability of CCK-8 to increase tyrosine phosphorylation of EGFR and ERK. Total EGFR and ERK are shown. (B) The % EGFR tyrosine phosphorylation mean value ± S.E. of 3 experiments is indicated; $p < 0.05$, *; $p < 0.01$, ** relative to control; $p < 0.01$, [aa]: relative to CCK-8 using the Student's t-test. (C) The % ERK tyrosine phosphorylation mean value ± S.E. of 3 experiments is indicated; $p < 0.05$, * relative to control; $p < 0.05$,[a]; relative to CCK-8 using the Student's t-test.
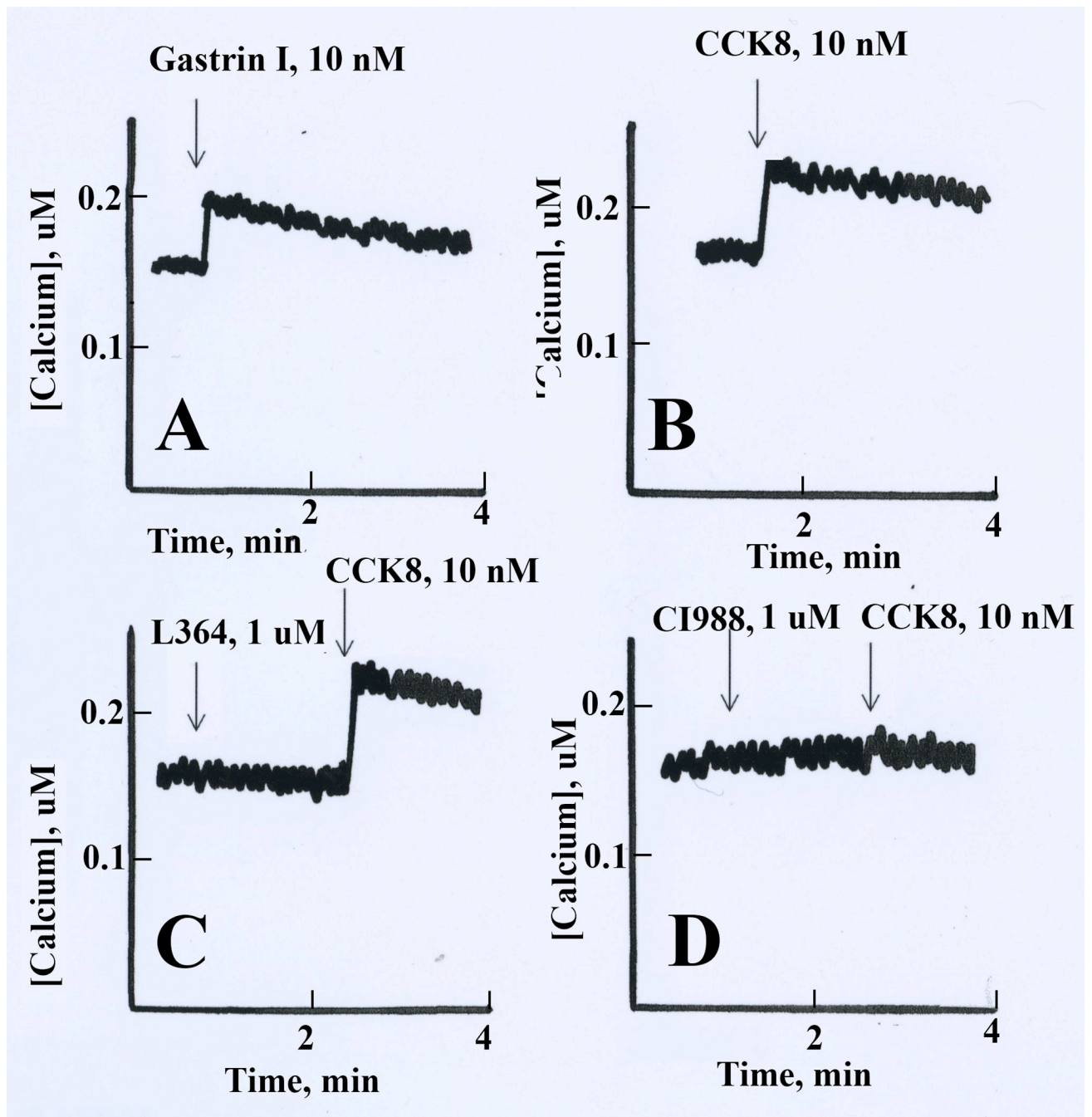
Fig. 3.
Cytosolic Ca^{2+}. The ability of gastrin-17 (A) and CCK-8 (B) to increase cytosolic in Fura-2AM loaded NCI-H727 cells is shown. (C) L364,718 (L364) had no effect on basal cytosolic Ca^{2+} or the ability of CCK-8 to increase cytosolic Ca^{2+}. (D) CI-988 had no effect on basal cytosolic Ca^{2+} but antagonized the ability of CCK-8 to increase cytosolic Ca^{2+}. This experiment is representative of 3 others.
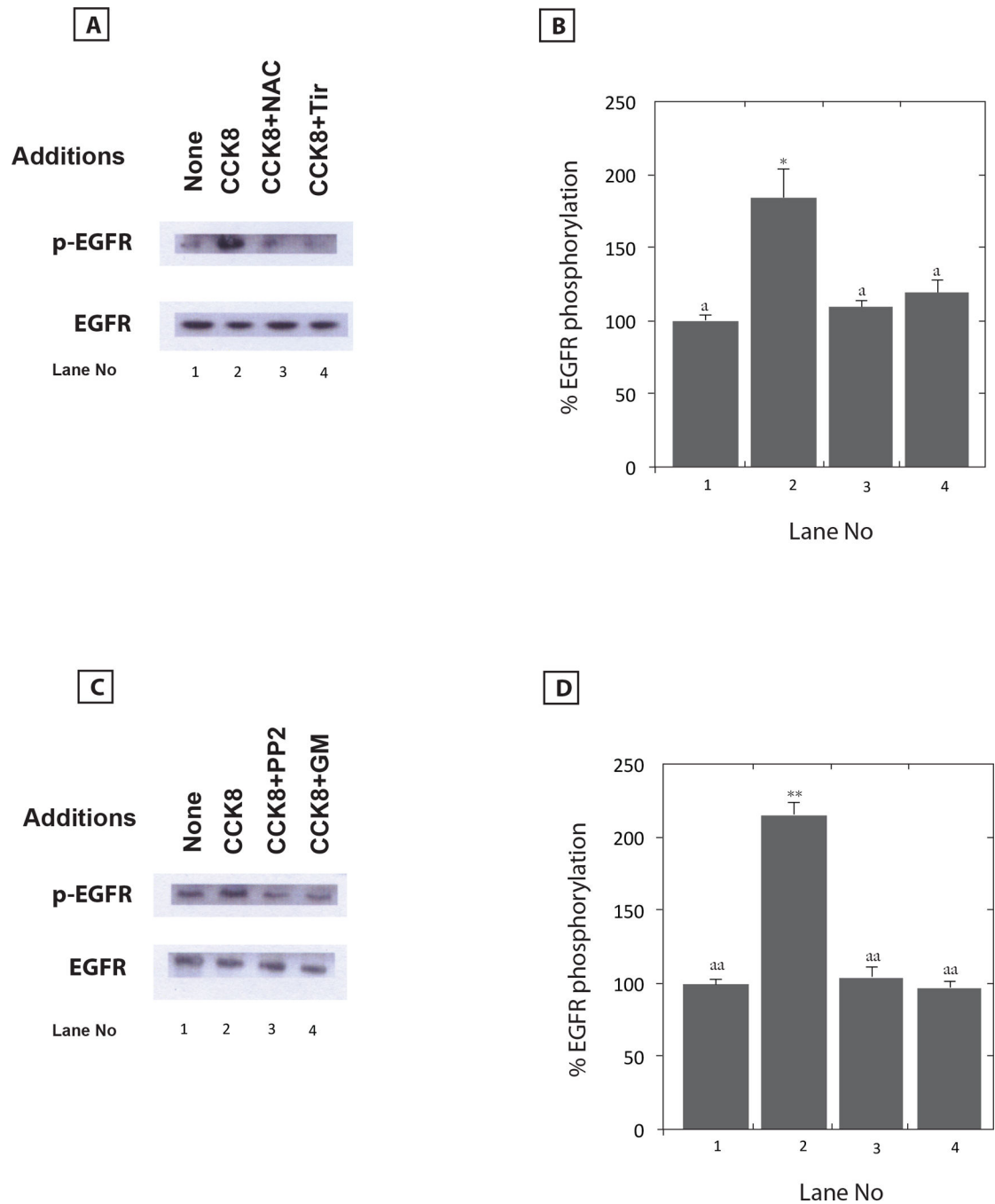
Fig. 4.
EGFR transactivation inhibitors. (A) Five mM NAC or Tiron (Tir) had no effect on basal EGFR tyrosine phosphorylation but inhibited the increase in EGFR transactivation caused by CCK8 addition to NCI-H727 cells. (B) The mean value ± S.E. of 3 experiments is indicated; $p < 0.05$, *; relative to control; ; $p < 0.05$,[a]; relative to CCK-8 using the Student's t-test. (C) Ten μM PP2 or GM6001 (GM) had no effect on basal EGFR tyrosine phosphorylation but inhibited the increase in EGFR transactivation caused by CCK-8

addition to NCI-H727 cells. (D) The mean value ± S.E. of 3 experiments is indicated; $p < 0.01$, ** relative to control; $p < 0.01$, aa: relative to CCK-8 using the Student's t-test.
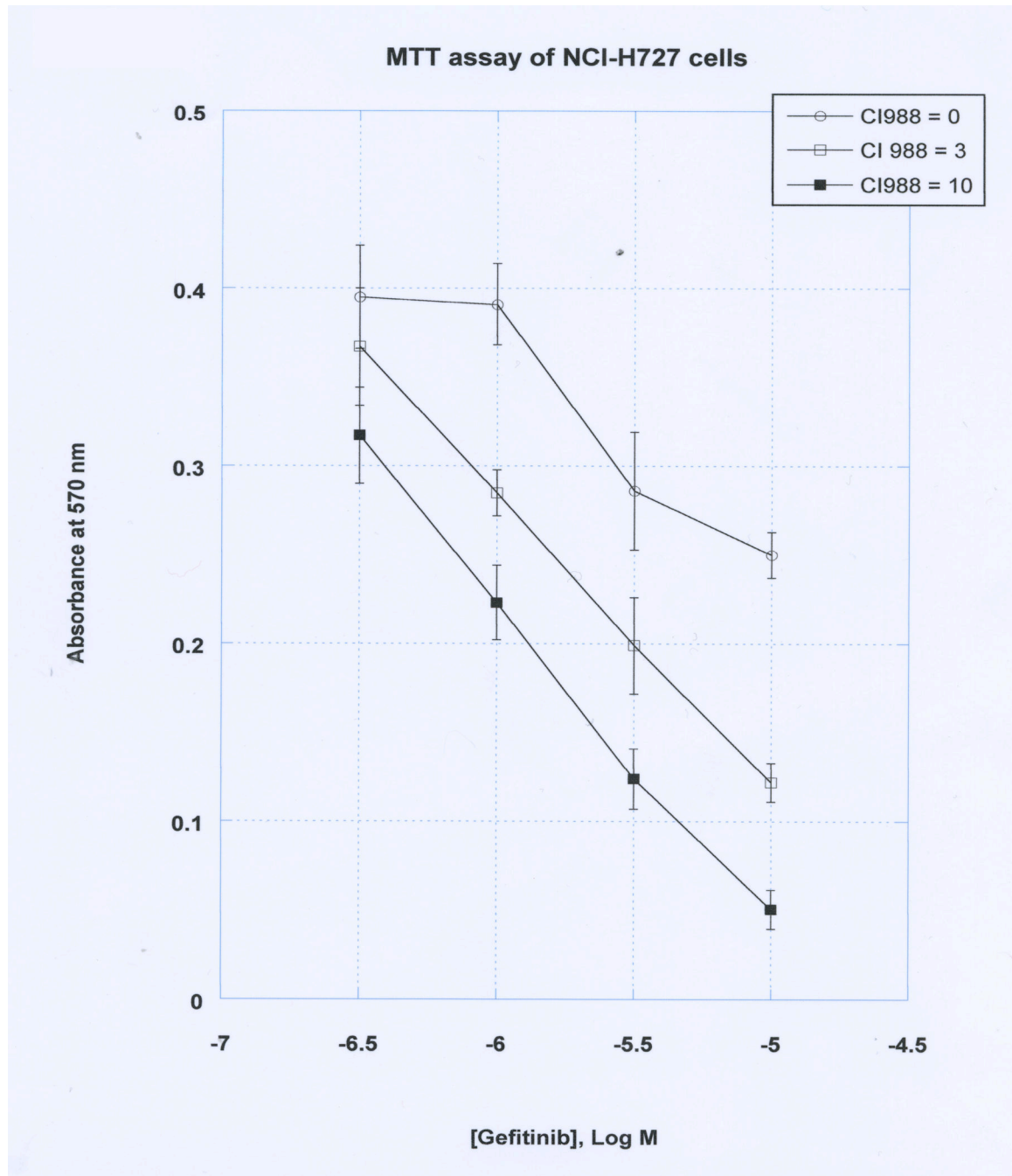
Fig. 5.

CI-988 and gefitinib growth inhibition. Varying doses of gefitinib were added with (○) no additions, (▫) 3 μM CI-988 and (▪) 10 μM-CI988 to NCI-H727 cells using the MTT assay. The mean value ± S.E. of 8 determinations is indicated. This experiment is representative of 3 others.

Table I

Binding of CCK analogs

Peptide	Ki, nM
CCK-8	0.8 ± 0.2
CCK-8NS	1.2 ± 0.3
Gastrin-17	5.9 ± 0.91
CCK-4	22 ± 4
CI-988	4.5 ± 0.9
Gefitinib	>1800

^{125}I-BH-CCK-8 was bound to NCI-H727 cells as a function of dose of unlabeled competitor and the Ki calculated. The mean value $\pm$ S.D. of 3 determinations is indicated. The structures of CCK-8 and gastrin-17 are shown below. Sequence homologies are underlined.

Gastrin-17	Met-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-<u>Gly-Trp-Met-Asp-Phe-NH$_2$</u>
CCK-8NS	Asp-Tyr-Met-<u>Gly-Trp-Met-Asp-Phe-NH$_2$</u>

Table II

Reactive oxygen species

Addition	Relative fluorescence
None	100 ± 5^a
CCK-8, 100 nM	$128 \pm 9^*$
CCK-8 + CI-988 10 uM	104 ± 6^a

NCI-H727 cells were treated with 100 nM CCK-8 for 60 min and the relative fluorescence determined. The mean value $\pm$ S.D. of 8 determinations is indicated; $p < 0.05$,

*
relative to control; $p < 0.05$,

a
relative to CCK-8 using the Student's t-test.

Table III

Clonal growth of NCI-H727 cells.

Addition	Colony number	Colony number + 10 nM CCK-8
None	42 ± 6	$74 \pm 9^{*}$
CI-988, 3 uM	$21 \pm 3^{*}$	44 ± 5
Gef, 1 uM	$26 \pm 4^{**}$	49 ± 7
CI-988 + Gef	$9 \pm 2^{**}$	$28 \pm 5^{*}$

The mean value ± S.D. of 3 determinations is indicated;

*
, $p < 0.05$;

**
, $p < 0.01$ relative to control using Student's t-test.