Metabotropic Glutamate Receptor 4-Mediated 5-Fluorouracil Resistance in a Human Colon Cancer Cell Line

Byong Chul Yoo,1 Eunkyung Jeon,1 Sung-Hye Hong,2 Young-Kyoung Shin,2 Hee Jin Chang,1 and Jae-Gahb Park1,2
1Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi, Korea, and 2Cancer Research Institute and Cancer Research Center, Seoul National University, Seoul, Korea

ABSTRACT

Purpose: 5-Fluorouracil (5-FU) has been the mainstay treatment for colorectal cancer for the past few decades. However, as with other cancers, development of 5-FU resistance has been a major obstacle in colorectal cancer chemotherapy. The purpose of this study was to gain further understanding of the mechanisms underlying 5-FU resistance in colorectal cancer cells.

Experimental Design: A 5-FU-resistant cell line was established from the human colon cancer cell line SNU-769A. Protein extracts from these two cell lines (parent and resistant) were analyzed using comparative proteomics to identify differentially expressed proteins.

Results: 5-FU-resistant human colon cancer cells were found to overexpress metabotropic glutamate receptor 4 (mGluR4). Other experiments showed cellular resistance to 5-FU (i.e., cell survival) was altered by the mGluR4 agonist L-2-amino-4-phosphonobutyric acid (L-AP4), and by the mGluR4 antagonist (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP 4), in that L-AP4 increased 5-FU resistance in SNU-769A cells, whereas MAP 4 ablated 5-FU resistance in 5-FU-resistant cells. However, there was no significant effect of L-AP4 or MAP 4 on basal cAMP and thymidylate synthase levels. Interestingly, 5-FU down-regulated mGluR4 expression, and MAP 4 suppressed proliferation in both cell lines.

Conclusions: We here report mGluR4 expression in human colon cancer cell line, which provides further evidence for extra-central nervous system expression of glutamate receptors. Overexpression of mGluR4 may tentatively be responsible for 5-FU resistance and, although activation by agonist promotes cell survival in the presence of 5-FU, decreased mGluR4 expression or inactivation by antagonist contributes to cell death.

INTRODUCTION

Colorectal cancer is one of the most common causes of cancer-related death in Western countries (1). In patients with advanced colorectal cancer, 5-year survival is quite poor (2). Treatment of advanced colorectal cancer is often complicated because of its tendency to be chemotherapy-resistant (3). Over the past 40 years, 5-fluorouracil (5-FU) has been almost the sole chemotherapeutic option for patients with advanced colorectal cancer and is still considered a mainstay of therapy (4). However, major responses after 5-FU treatment are observed in only about 10% of advanced colorectal cancer patients (4). Attempts to enhance the therapeutic effectiveness of 5-FU through biochemical modulation with methotrexate or leucovorin (5, 6) have resulted in higher response rates. For example, treatment of advanced colorectal cancer with 5-FU associated with leucovorin resulted in a remission rate of 23% compared with 11% for patients treated with 5-FU alone (6). However, patients who respond to 5-FU ultimately become resistant, and 5-FU/leucovorin has produced only a small increase in survival rate (7, 8).

As is the case in the treatment of other cancer types, resistance to 5-FU has been a major obstacle in advanced colorectal cancer chemotherapy (9, 10).

Various mechanisms have been proposed to explain resistance to 5-FU. 5-FU resistance has been correlated with either defective drug uptake or alterations in the activities of anabolic and/or catabolic enzymes (11–13). Several other mechanisms of resistance to 5-FU have been attributed to thymidylate synthase (TS), which is a crucial enzyme in de novo synthesis of thymidylate (14). For example, 5-FU resistance appears linked to altered binding of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) to TS, TS gene amplification, and TS gene mutations (15–17).

Efforts to overcome 5-FU resistance are essential because it still remains the agent of choice for colorectal cancer treatment. The evaluation of additional parameters such as other (fluoro)pyrimidine-converting enzymes, and the mutation status of regulators of apoptosis and tumor angiogenesis are also currently under investigation (18).

In the present study, we sought to gain further insight into the mechanisms underlying 5-FU resistance. We established a 5-FU-resistant human colon cancer cell line and used these cells in comparative proteomics studies. We found that 5-FU resistance was associated with overexpression of metabotropic glutamate receptor 4 (mGluR4).

MATERIALS AND METHODS

5-FU-Resistant Human Colon Cancer Cell Line. The human colon cancer cell line SNU-769A (19) was obtained from the Korean Cell Line Bank (Seoul, Korea). A cell line resistant...
to 5-FU (Choongwae Pharma Corporation, Gyeonggi, Korea) was derived from these cells, as described previously (20). Briefly, cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% bovine calf serum, 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies, Inc.). The initial 5-FU concentration added to cells was 10% of the IC_{50} (defined as the concentration producing a 50% reduction in absorbance at 540 nm compared with untreated controls) and 0.05% Tween 20-Tris-buffered saline solution (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg each of pepstatin A, chymostatin, leupeptin, and antipain) with a Potter-Elvehjem homogenizer at 4°C. Protein concentration of homogenates was estimated using the dye-binding method (22) with BSA as a standard. Homogenates were centrifuged at 4000 × g for 10 min at 4°C, and supernatants containing equivalent amounts of protein were subjected to Laemmli SDS-PAGE (26). After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), which were then blocked with 1% bovine serum albumin in TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). Membranes were then incubated with 0.1% Tween 20-Tris-buffered saline containing 1.5% nonfat dry milk (Bio-Rad, Hercules, CA) and 1 mM MgCl2. Membranes were then incubated for 2 h at room temperature with diluted primary antibodies against either mGlur4 (Upstate Biotechnology, Lake Placid, NY), TS (Chemicon, Temecula, CA), poly(ADP-ribose)polymerase (abcam, Cambridge, Cambridgeshire, United Kingdom) or β-actin (Sigma). Membranes were then washed three times for 15 min each with blocking solution and incubated with diluted horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were then washed again three times for 15 min with blocking solution and incubated with WEST-ZOL (plus) chemiluminescence reagent (iNtRON Biotechnology, Seoul, Korea) for 1 min and exposed to film (Kodak Blue XB-1, Rochester, NY).

Measurement of cAMP. cAMP levels were determined using a cAMP enzyme immunoassay kit (Sigma), according to the manufacturer’s instructions. Briefly, cells growing in suspension were harvested and washed thoroughly with PBS to remove extracellular cAMP. Cells resuspended in PBS were divided into two equal volumes that were used for either protein or cAMP determination. For cAMP determination, cells were incubated with 0.1 mM HCl for 10 min and centrifuged at 1000 × g at room temperature, and the supernatant was used directly for cAMP measurement. To this supernatant was added cAMP polyclonal rabbit antibody and alkaline phosphatase conjugated with cAMP, and this mixture was added to microtiter plates coated with goat antirabbit IgG and was incubated for 2 h at room temperature with shaking. The wells were washed three times, p-nitrophenyl phosphate solution was added, and the
wells were incubated for 1 h at room temperature without shaking, after which the reaction was stopped by the addition of trisodium phosphate solution. Absorbance was then measured at 405 nm and was used to determine the cAMP concentration (pmol/ml), which was then used to determine pmol cAMP per mg cell protein.

**Statistical Analysis.** The nonparametric Mann-Whitney U test was used to evaluate the differences between groups, and statistical significance was set at a \( P \) value of <0.05. Synergism was assessed using the method of Chou and Talalay (27) with a nonfixed ratio experimental design and Calcusyn software (Biosoft, Ferguson, MO). A combination index value \(<1.0\) indicates synergism.

**RESULTS**

Establishment of 5-FU-Resistant Cell Line from Human Colon Cancer Cell Line SNU-769A. A 5-FU-resistant cell line was established from the human colon cancer cell line SNU-769A as described in the “Materials and Methods.” The \( IC_{50} \) for 5-FU was 3-fold higher in resistant cells (1.48 ± 0.10 \( \mu \)g/ml) compared with parent cells (0.42 ± 0.08 \( \mu \)g/ml). The 5-FU-resistant cell line did not exhibit cross-resistance to cisplatin, with the cisplatin \( IC_{50} \) in parent and resistant cells being 1.40 ± 0.53 \( \mu \)g/ml and 1.39 ± 0.38 \( \mu \)g/ml, respectively.

**Overexpression of mGluR4 in 5-FU-Resistant Cells.** Proteins from SNU-769A parent and 5-FU-resistant cells were analyzed using 2-DE (Fig. 1A). Image analysis after Coomassie Blue staining revealed a spot with a molecular size (\( M_r \)) of 110,000 and isoelectric point (\( pI \)) 7.5, which was more highly expressed in resistant cells compared with parent cells. The spot was excised, digested by trypsin, and peptide masses were determined using MALDI-MS analysis. A Swiss-Prot database search identified the protein as metabotropic glutamate receptor 4 (mGluR4) precursor (Table 1). The overexpression of mGluR4 in resistant cells was confirmed using Western blot analysis with an antibody against mGluR4 (Fig. 1B). Testing of
cell/tissue homogenates from other sources showed mGluR4 was present in mouse brain extracts but not in Huh7 human hepatocarcinoma cell line extracts (Fig. 1B).

Effect of mGluR4 Ligands on cAMP Level and Resistance to 5-FU. The basal level of cAMP in 5-FU resistant cells (4.02 pmol/mg protein) was found to be significantly lower than that in parent cells (7.98 pmol/mg protein; \( P = 0.0286 \); Fig. 2A). Whereas the addition of 25 \( \mu \)M forskolin increased cAMP levels by about 20-fold in parent cells (150.96 pmol/mg protein), the increase in resistant cells was only 10-fold (35.04 pmol/mg protein; Fig. 2B). This forskolin-induced increase in cAMP levels was inhibited by the mGluR4 agonist L-2-amino-4-phosphonobutyric acid (L-AP 4) in parent cells but not in resistant cells (Fig. 2B). Neither L-AP 4 (Fig. 3A) nor MAP 4 (Fig. 3B) was able to alter basal cAMP levels in the absence of forskolin in either cell. Furthermore, parallel experiments also failed to show any L-AP 4- or MAP 4-mediated changes in T3 protein expression in either cell (Fig. 3). By contrast, these two mGluR4 ligands affected cell death in response to 5-FU. In the presence of L-AP 4 or MAP 4, 5-FU was added to cells, and survival was measured at the time indicated using an MTT assay (Fig. 4). At 24 h after treatment, the difference in parent cell survival between cells treated with 5-FU plus L-AP 4 and cells treated with 5-FU alone was significant (\( P = 0.0286 \); Fig. 4A, right panel). The MAP 4 synergism with 5-FU in 5-FU-resistant cells had a combination index value of 0.630 (Fig. 4B, right panel).

Down-Regulation of mGluR4 Expression by 5-FU and Cytotoxicity of MAP 4. Addition of 5-FU was associated with decreased mGluR4 protein expression in both cell lines, although the extent of the decrease differed between the two cell lines (Fig. 5A). 5-FU decreased mGluR4 expression time- and dose-dependently in parent cells. Although a similar decrease was noted in resistant cells, the decrease was observed only in the presence of 4.20 \( \mu \)g/ml 5-FU.

Cisplatin was investigated to determine whether other anticancer drugs also affected mGluR4 expression. We found cisplatin caused neither time- nor dose-dependent changes in mGluR4 expression in either cell line (Fig. 5B).

Proliferation of both parent and resistant cells was suppressed by about 20% at 12 h after the addition of 1 \( \mu \)M L-AP 4 (Fig. 5C). MAP 4 was a more powerful inhibitor, with 1 \( \mu \)M MAP 4 significantly suppressing proliferation in both cell lines by about 50% at 12 h after the addition (Fig. 5D). Although 0.1 \( \mu \)M MAP 4 had a more pronounced inhibitory effect on resistant cells compared with parent cells at 3 and 6 h after the addition, the overall pattern of proliferation suppression did not differ between parent and resistant cells at the times shown (Fig. 5D). Western blot analysis showed that cleavage of intact poly(ADP-ribose) polymerase to the 89 kDa fragment, typical of apoptotic cell death, was induced in both cell lines at 12 h after treatment with MAP 4 (Fig. 5D, bottom panels). These results demonstrate that MAP 4 induced apoptosis in both cell lines.

### Table 1 MALDI-MS analysis of the protein overexpressed in 5-FU-resistant cells

<table>
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<th>SWISS-PROT No.</th>
<th>Abbreviation name</th>
<th>Full name of protein</th>
<th>Theor. M_r</th>
<th>Observ. M_r</th>
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<td>Q14833</td>
<td>MGR4_HUMAN</td>
<td>Metabotropic glutamate receptor 4 precursor (mGluR4)</td>
<td>103,350</td>
<td>110,000</td>
<td>8.96</td>
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</table>

* MALDI-MS, matrix-associated laser desorption ionization-mass spectrometry; 5-FU, 5-fluorouracil.
In the absence of forskolin, no effect of L-2-amino-4-phosphonobutyric acid (L-AP 4; A) and (S)-amino-2-methyl-4-phosphonobutanoic acid (MAP 4; B) on the intracellular cAMP concentration and thymidylate synthase (TS) protein expression. Cells incubated with either L-AP 4 or MAP 4 for 24 h were harvested and were used for the determination of intracellular cAMP content and Western blot analysis for TS protein expression. In the absence of forskolin, neither L-AP 4 nor MAP 4 was able to alter basal cAMP level or TS protein expression in either cell. Different incubation time also failed to show L-AP 4- or MAP 4-mediated changes in either cell (data not shown). Data are means ± SDs from three independent experiments.

Fig. 4 Changed 5-fluorouracil (5-FU) resistance by L-2-amino-4-phosphonobutyric acid (L-AP 4) or (S)-amino-2-methyl-4-phosphonobutanoic acid (MAP 4). A, in the presence of L-AP 4, 5-FU was added to cells, and survival was measured at the times indicated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay; ○, none; ▲, L-AP 4 (100 μM); ○, 5-FU (0.21 μg/ml); △, L-AP 4 + 5-FU. B, in the presence of MAP 4, 5-FU was added to cells and survival was measured at the times indicated using an MTT assay; ○, none; ■, MAP 4 (0.1 μM); ○, 5-FU (4.20 μg/ml); □, MAP 4 + 5-FU. At 24 h after treatment, the difference in parent cell survival between cells treated with 5-FU plus L-AP 4 and cells treated with 5-FU alone was significant (P = 0.0286; A, right panel). The MAP 4 synergism with 5-FU in 5-FU-resistant cells had a combination index value of 0.630 (B, right panel). Data are means ± SDs from four independent experiments.
DISCUSSION

mGluR4 Overexpression in a 5-FU-Resistant Human Colon Cancer Cell Line. Although drug resistance in cancer chemotherapy is a common phenomenon, knowledge about the mechanisms underlying this resistance is far from complete. Here we used comparative proteomics to identify proteins that might be involved in 5-FU resistance in colon cancer. 2-DE, MALDI-MS, and Western blotting studies (Fig. 1, A–C) revealed that mGluR4 was expressed at higher levels in a 5-FU-resistant human colon cancer cell line compared with its expression in the parent SNU-769A cells, which originated from mucinous adenocarcinomas (19). mGluR4 was also detected in the mouse brain extract positive control, but not in the Huh7 human hepatoma cell line (25). 2-DE and MALDI-MS analysis showed that other proteins were also overexpressed in resistant cells, namely Q12931, heat shock protein 75 kDa, mitochondrial precursor (HSP 75), and Q13228, selenium binding protein-1.

2-DE gel analysis showed that for the vast majority of cell...
proteins there was no difference in expression between the two cell lines. This was not surprising as the cell homogenates contain large proportions of housekeeping and structural proteins. However, these assays have identified additional proteins that appear to be differentially expressed, and we are currently seeking to confirm these findings using other techniques (i.e., Western blotting and enzyme activity assays).

mGluRs are G-protein coupled receptors that have been subdivided into three groups based on sequence similarity, pharmacology, and intracellular signaling mechanisms (28). Group I mGluRs (mGluR1 and mGluR5) are coupled to phospholipase C and intracellular calcium signaling, and group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) are negatively coupled to adenylyl cyclase (28, 29). Although mGluRs are implicated in regulation of diverse neuronal plasticity and neuropathological processes in the central nervous system, the expression of these receptors in both normal and cancer cells outside of the central nervous system is beginning to be appreciated (30–33). For example, mGluR8 in rat pancreatic islets (30) and group I and II mGluRs in rat heart (31) have been reported. Furthermore, bone osteoblasts and osteoclasts, megakaryocytes, keratinocytes, taste buds, and cells in the lung, heart, kidney, and adrenal glands possess glutamate receptors (32). Recently, Pollock et al. (33) detected mGluR1 in human melanoma biopsies and in melanomas obtained from transgenic mice predisposed to develop multiple melanoma, but they failed to detect increased mGluR1 expression in normal human and murine melanocytes. Transgenic mice with mGluR1 expression were more susceptible to melanoma, providing compelling evidence for the importance of mGluR1 signaling in melanocytic neoplasia (33). These findings suggest mGluRs have a role in regulating the physiology of both normal and cancer cells in organs outside the central nervous system.

Our findings correlating mGluR4 expression with 5-FU
resistance are consistent with those of Pollock et al. (33) and suggest a physiological role for mGluR4 in human colon cancer.

**mGluR4 Negatively Coupled to Adenylate Cyclase.** Group II and III mGluRs down-regulate the adenylate cyclase and cAMP cascades (29), and it is known that the mGluR4 agonist L-AP 4 inhibits forskolin-stimulated cAMP production (29, 34). We found the basal intracellular cAMP concentration was significantly lower in 5-FU-resistant cells compared with parent cells (P = 0.0286; Fig. 2A). Addition of forskolin, although quantitatively increased basal level of cAMP in both cell lines, the real difference remained the same, with the increase being 20-fold in case of parent and 10-fold in resistant cell line (Fig. 2B). The 50% cAMP levels in resistant compared with parent cell line in basal condition could be attributed to a relatively low activity of adenylate cyclase that was probably caused by overexpression of mGluR4. Forskolin-induced cAMP production was decreased by the addition of L-AP 4 in parent cell line (Fig. 2B). This finding is not only consistent with the negative coupling of mGluR4 (a group III mGluR) to adenyl cyclase but also suggests that this pathway might be operative in human colon cancer cell line SNU-769A. The finding of unchanged forskolin-induced cAMP production by L-AP 4 in 5-FU-resistant cells despite overexpressed mGluR4 may be explained by agonist-induced receptor down-regulation because this is not an uncommon phenomenon for ligand-receptor interaction. Thus, it can be presumed that, although overexpression of mGluR4 per se is necessary and sufficient for the inhibition of adenyl cyclase, this inhibition can be overcome in the presence of sufficient ligand.

**Modulation of 5-FU Resistance by a mGluR4 Agonist and Antagonist.** Because mGluR4 was up-regulated in 5-FU-resistant cells, we investigated the possible role of mGluR4 on resistance patterns using an mGluR4 agonist and antagonist. Interestingly, the mGluR4 agonist L-AP 4 increased 5-FU resistance in SNU-769A (relative survivals in cells treated with 5-FU alone and 5-FU/L-AP 4 at 12 h after treatment were 64.13 ± 0.78% and 84.69 ± 5.92%, respectively; P = 0.0286), whereas the mGluR4 antagonist MAP 4 acted synergically with 5-FU to enhance death in 5-FU-resistant cells highly expressing mGluR4 (combination index value of MAP 4 and 5-FU was 0.630; Fig. 4B). This result is consistent with the findings of Rzeski et al. (35), who reported synergy between glutamate antagonists and common cytostatic drugs used in cancer therapy. The N-methyl-D-aspartate (NMDA) antagonist dizocilpine and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) antagonist GYKI52466, potentiated the efficacy of the cytostatic drugs cyclophosphamide and thiotepa in human rhabdomyosarcoma/medulloblastoma and human neuroblastoma cell lines. These findings imply that better cytostatic effects might be achieved through the combined use of glutamate antagonists and existing chemotherapeutic regimens.

To investigate whether mGluR4-mediated 5-FU resistance was directly linked to the modulation of intracellular cAMP levels, we examined the effect of L-AP 4 and MAP 4 in the absence of forskolin on intracellular cAMP. We found neither L-AP 4 nor MAP 4 affected intracellular cAMP levels in either parent or resistant cells (Fig. 3). Thus, even though mGluR4 is a well-known regulator of cAMP formation (via negative control of adenylate cyclase activity), the results suggest that neither adenylate cyclase nor TS was involved in mGluR4-mediated 5-FU resistance (Fig. 3).

**Decreased mGluR4 Expression by 5-FU.** To better understand how mGluR4 mediates 5-FU resistance, we measured mGluR4 expression in the presence of 5-FU. Interestingly, 5-FU down-regulated mGluR4 expression time- and dose dependently in the parent cell line (Fig. 5A). The decrease in mGluR4 expression in 5-FU-resistant cells was observed only in the presence of 4.20 μg/ml 5-FU (Fig. 5A), and cisplatin failed to decrease expression in either cell line (Fig. 5B). Although these data do not allow us to identify how 5-FU affects mGluR4 expression, they do indicate a link between mGluR4 expression and 5-FU resistance.

The mGluR4 antagonist MAP 4 (Fig. 5D) was cytotoxic, whereas L-AP 4 lacked any significant cytotoxicity or effect on cell proliferation (Fig. 5C). MAP 4 cytotoxicity in both cell lines increased as its concentration was raised. A different response to MAP 4 was observed in 5-FU-resistant cells at 3 and 6 h after treatment with 0.1 μM MAP 4. Early responses to MAP 4 appeared to occur in 5-FU-resistant cells with an increased mGluR4 expression. However, overall MAP 4-induced suppression of cell proliferation did not differ between parent and 5-FU-resistant cells at the times shown in Fig. 5D. These findings are consistent with those of Rzeski et al. (35), which show the inhibitory effect of glutamate antagonists on the proliferation of human tumor cells, including colon adenocarcinoma, astrocytoma, and breast and lung carcinoma cells. Proliferation of tumor cells derived from peripheral (non-central nervous system) tissues is more sensitive to suppression by glutamate antagonists compared with those of central nervous system origin (36). This effect is potentially important in that there are many glutamate receptor antagonists already available that do not readily penetrate blood–brain barriers, and such agents can be used in relatively high concentration to treat peripheral cancers without inducing adverse neurological side effects. This characteristic suggests glutamate receptor antagonists may be useful as adjuvants in cancer chemotherapy.

There is an increasing body of evidence indicating the pivotal role of mGluR4 signaling in the protection of cells. For example, decreased expression of the mGluR4 gene is associated with neuron apoptosis (37), and selective activation of mGluR4 is protective against excitotoxic neuron death (37). Furthermore, the activation of microglial group III mGluRs protects neurons against microglial neurotoxicity (38). Consistent with these findings, the present data suggest the mechanism underlying 5-FU resistance in human cancer cells involves increased expression of mGluR4 (Fig. 1). Another factor that may contribute to cellular resistance against 5-FU-induced cell death is that mGluR4 expression in the resistant cell line was less affected by the addition of 5-FU compared with the parent cells (Fig. 5A).

In summary, the present study reports on the mGluR4-mediated 5-FU resistance in human colon cancer cells. The results demonstrate that elevated mGluR4 expression or activation by agonists promotes cell survival in the presence of 5-FU, whereas decreased mGluR4 expression or inactivation by antagonists contributes to cell death. Cancer cells overexpressing mGluR4 to protect against 5-FU cytotoxicity may become 5-FU resistant. Our data suggest increased mGluR4 expression is a
mechanism underlying 5-FU resistance in human colon cancer cells.

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