Etodolac Improves 5-FU Sensitivity of Head and Neck Cancer Cells through Inhibition of Thymidylate Synthase

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Abstract. *Background: 5-Fluorouracil (5-FU) is widely used in the treatment of head and neck squamous cell carcinoma (HNSCC). However, development of drug resistance is one of the major causes of HNSCC treatment failure. The goal of this study was to investigate the mechanism of 5-FU resistance and to develop a novel combination therapy with another agent which sensitizes cells to 5-FU. Materials and Methods: A 5-FU-resistant cell line, UM-SCC-23F/R, was developed from UM-SCC-23 cells. We determined sensitivities to 5-FU, etodolac and a combination treatment and also analyzed the expressions of cyclooxygenase-2 (COX-2) and thymidylate synthase (TS). Results: Selective COX-2 inhibitor, etodolac, sensitized UM-SCC-23F/R cells to 5-FU. Expression of COX-2 decreased after etodolac treatment in both cell lines. While overexpression of TS was observed in UM-SCC-23F/R cells, etodolac inhibited TS expression, suggesting that the sensitizing effect induced by etodolac depends on TS suppression. Conclusion: We demonstrate for the first time an important inhibitory effect of etodolac on TS expression leading to sensitization to 5-FU in 5-FU-resistant cells. Our data suggest that TS inhibition can be accomplished by this routinely used nonsteroidal anti-inflammatory drug, and this may have a role as novel effective cancer treatment for 5-FU-resistant cancer.*

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5-Fluorouracil (5-FU) is an anti-metabolite widely used in the treatment of head and neck squamous cell carcinoma (HNSCC) in combination with cisplatin and docetaxel (1). This agent is also used as an oral drug such as tegafur-uracil (UFT) and TS-1, in head and neck carcinoma treatment (2). However, drug resistance contributes to therapy failure in many cases (3). Although there have been many studies on the mechanisms of chemoresistance, effective strategies against 5-FU resistance in HNSCC have not been identified.

The anticancer effect of 5-FU is *via* its incorporation into DNA and RNA, inducing cell cycle arrest and apoptosis (4). Thymidylate synthase (TS), which is the rate-limiting enzyme in *de novo* DNA biosynthesis, catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine (dTMP), an essential step in DNA biosynthesis. A metabolic byproduct of 5-FU, 5,10-methylenetetrahydrofolate,5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), forms a slowly reversible covalent complex with TS, thereby blocking the synthesis of DNA. TS plays an important role in cancer cells acting as a molecular target of 5-FU. It has been shown that higher expression levels of TS confer lower sensitivity to 5-FU in head and neck, gastrointestinal and colon cancer (5). This suggests that the TS expression level may serve as a biomarker for 5-FU resistance.

In order to improve the effectiveness of 5-FU therapy, different drugs and modifiers have been studied. Overcoming

5-FU resistance was seen *via* cyclooxygenase-2 (COX-2) inhibition in liver and renal cancer (6). COX-2 is induced by a variety of substances including pro-inflammatory cytokines, growth factors, mitogenic substances, oncogenes, and hypoxia (7). COX-2 catalyzes the production of prostaglandins, such as PGE-2 and PGI-2, resulting in vasodilatation and pain. Interleukin-1 beta (IL-1β) is one of several cytokines known to potently induce COX-2 in a variety of cells (8). Overexpression of COX-2 has been associated with resistance to conventional therapies and overall poor prognosis in several types of cancer (9-12). COX-2-selective inhibitors have been shown to induce apoptosis in solid tumor cell lines, including glioma, head and neck, esophageal, lung, pancreatic, uterine cervical, prostatic and colon cancer (13). Etodolac, a selective inhibitor of COX-2, is widely used to treat patients with inflammatory pain as a non steroidal anti-inflammatory drug (NSAID), and also may have potential clinical applications as a preventative and therapeutic cancer drug (14).

The aim of this study was to elucidate the mechanisms of 5-FU resistance and improve sensitivity to 5-FU by using a COX-2-selective inhibitor in head and neck carcinoma cells.

Materials and Methods

Chemicals. Etodolac (Nippon Shinyaku, Kyoto, Japan) was dissolved in dimethyl sulfoxide (DMSO) and was then diluted with Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, CA, USA). IL-1β (Bachem AG, Switzerland) was diluted in 10% FBS- Minimum Essential Medium Alpha Modification (Sigma).

Cells and cell culture. UM-SCC-23 human HNSCC cells were kindly provided by Dr. Thomas E. Carey, from the Department of Head and Neck Cancer Biology at the University of Michigan. This cell line was maintained in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37˚C.

Isolation of 5-FU-resistant cells. UM-SCC-23 cells (1.0×10^6) were inoculated into a 10-cm dish and cultured for 24 h in DMEM with 10% FBS. Cells were then treated with 5-FU (Kyowahakkou, Tokyo, Japan) at a concentration of 0.5 mg/ml for 24 h. After that, cells were cultured in DMEM without 5-FU until they returned to stable growth. Cells were then again treated with 5-FU, with the concentration of 5-FU treatment being raised step-wise from 1.0 to 48.0 mg/ml.

Cell viability assay. The cellular sensitivity to 5-FU and etodolac were determined by tetrazolium dye (MTT) cytotoxicity assay. Cells were plated in 48-well plates at 2.0×10^4 cells/well and allowed to adhere for 24 h. The medium was then changed to fresh medium with 5-FU, etodolac, and/or IL-1β and incubated for 48 h. The medium was then removed and replaced with 10% FBS-DMEM. Cell survival was then determined using Cell Proliferation Kit I (MTT) (Roche Applied Science, Mannheim, Germany). Each experimental data point represents the average of six replicates, and experiments were repeated at least three times.

Drug interaction between etodolac and 5-FU was assessed at a concentration ratio of 1:1, using the combination index (CI), where CI<1, CI=1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively (15). On the basis of the isobologram analysis for mutually exclusive effects, the CI value was calculated as follows: $CI=(D)_1/(Dx)_1+(D)_2/(Dx)_2$, where $(Dx)_1$ and $(Dx)_2$ are the concentrations of individual drugs etodolac and 5-FU, respectively, required to inhibit cell growth by 50% and $(D)_1$ and $(D)_2$ are the drug concentrations in combination treatments that also inhibit cell growth by 50% (isoeffective as compared with the single drugs).

Western blot analyses. To determine the expression levels of COX-2 and TS, cells were treated with etodolac and/or IL-1β for 48 h. After washing twice with PBS, cells were treated with RIPA solution (1% NP-40, sodium deoxycholate and 0.05% SDS in PBS). Thirty microgram of total lysate was separated on a 10% gel using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane after electrophoresis. The primary antibodies were mouse polyclonal anti-human COX-2 (Cayman Chemical Company, MI, USA), polyclonal anti-human TS (Millipore, MA, USA), polyclonal anti-β-actin (Santa Cruz, CA, USA) and were used at 1:3000, 1:3000, and 1:15000 dilutions, respectively. The secondary antibody was a peroxidase-conjugated anti-mouse IgG used at a 1:20000 dilution. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis. Data obtained represent the mean values of at least three different experiments and are expressed as the mean±SD. Statistical significance, set at $p<0.05$, was determined using *t*-tests.

Results

Etodolac enhances 5-FU sensitivity. In order to analyze the mechanism of 5-FU resistance in HNSCC, we isolated a 5-FU-resistant cell line from UM-SCC-23 human tongue carcinoma cells. We then assessed the 5-FU sensitivity of UM-SCC-23 and UM-SCC-23F/R cells using MTT assay (Figure 1 and Table I). As shown in Figure 1A, UM-SCC-23F/R cells were resistant to 5-FU compared to UM-SCC-23 cells. The IC_{50} of 5-FU in UM-SCC-23F/R cells was approximately 2.7-fold higher than that of UM-SCC-23 cells ($p<0.01$). Etodolac was cytotoxic in a dose-dependent manner, although there was no significant difference in sensitivity between the cell lines (Figure 1B). The combination treatment of 5-FU and etodolac showed higher antitumor activity compared with both 5-FU and etodolac treatment alone (Figure 2A). The CI at IC_{50} was <1 at all concentrations of etodolac for UM-SCC-23 cells, indicating that simultaneous treatment of etodolac and 5-FU mediated a synergistic antitumor effect (Figure 2B). Interestingly, differential sensitivity of 5-FU between UM-SCC-23 and UM-SCC-23F/R cells disappeared when 5-FU was combined with etodolac treatment (Figure 1C). These results suggest that the COX-2 inhibition abolishes 5-FU resistance in the resistant cell line.

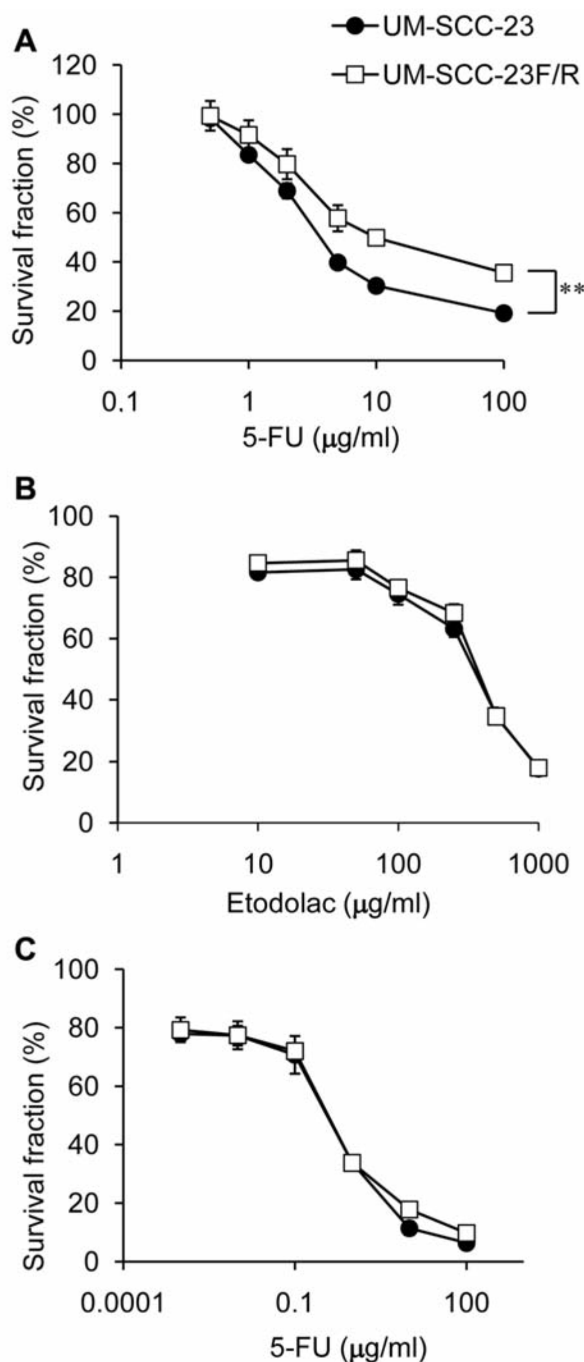

Figure 1. *A: Sensitivity of UM-SCC-23 and UM-SCC-23F/R cells treated with 5-FU. Dose-dependent effect of 5-FU on survival in both cell lines. Each data point is the mean of three independent experiments. Error bars show standard deviations. B: Sensitivity of UM-SCC 23 and UM-SCC 23 F/R cells treated with etodolac. Dose-dependent effect of etodolac on survival in both cell lines. Each data point is the mean of three independent experiments. Error bars show standard deviations. C: Sensitivity of UM-SCC-23 and UM-SCC-23F/R cells to treatment with 5-FU and etodolac (50 µg/ml). Dose-dependent effect of 5-FU and etodolac on survival in both cell lines. Each data point is the mean of three independent experiments. Error bars show standard deviations. **p<0.01, t-test.*

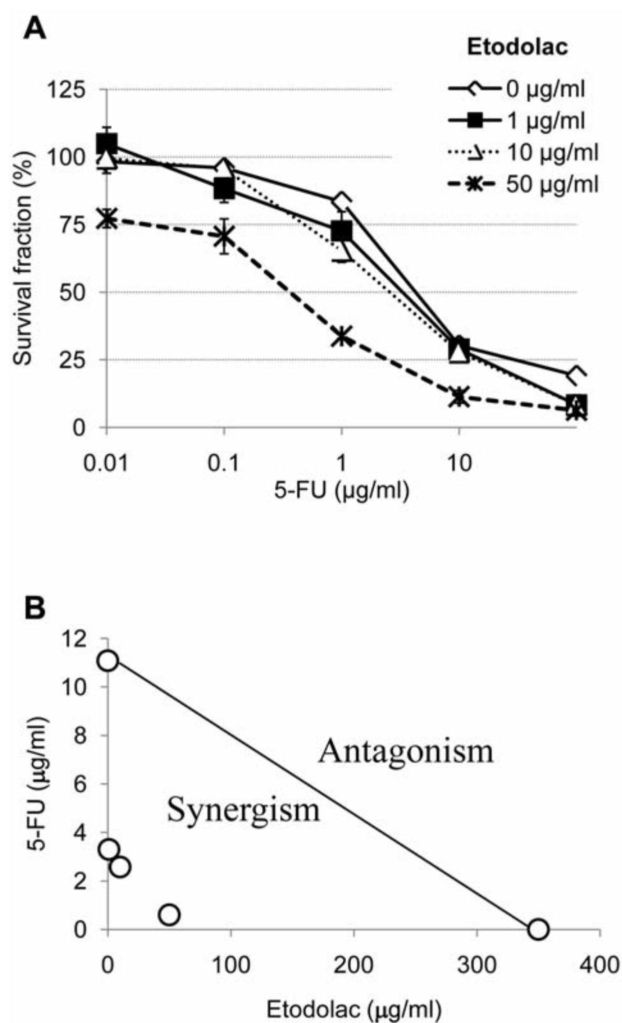

Figure 2. *A: Cytotoxic activity of 5-FU and etodolac combinations against UM-SCC-23 cells. Data shown are mean of triplicate determinations, with error bars representing SD. Experiments were repeated at least two times with similar results. B: Isobologram analysis of the data shown in (A). The IC_{50} values of each drug are plotted; the solid line represents an additive effect, while the values of the combination below this line indicate synergism of the combination.*

Table I. *IC_{50} of UM-SCC-23 and UM-SCC-23F/R cells for each agent.*

Agent	IC_{50}		*P*-value
	UM-SCC-23	UM-SCC-23F/R	
5-FU	3.63±0.85 (µg/ml)	9.86±1.44 (µg/ml)	<0.01
Etodolac	0.35±0.04 (mg/ml)	0.36±0.02 (mg/ml)	>0.05
5-FU+ Etodolac (50 µg/ml)	0.62±0.12 (µg/ml)	0.62±0.09 (µg/ml)	>0.05

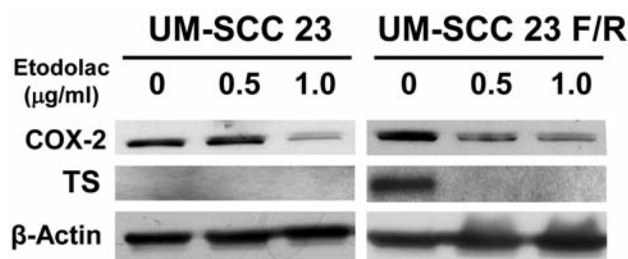

Figure 3. *Western blot analysis of cyclooxygenase-2 (COX-2) and thymidylate synthase (TS) expression on treatment of UM-SCC-23 and UM-SCC-23F/R cells with etodolac.*

Table II. *IC_{50} of UM-SCC-23 and UM-SCC-23F/R cells for each agent in the presence of IL-1β (1 ng/ml).*

Agent	IC_{50}		*P*-value
	UM-SCC-23	UM-SCC-23F/R	
5-FU	6.92±1.43 (μg/ml)	11.09±1.83 (μg/ml)	<0.01
5-FU+ Etodolac (50 μg/ml)	1.29±0.24 (μg/ml)	1.41±0.24 (μg/ml)	>0.05

Etodolac treatment down-regulates COX-2 and TS expression. Next we confirmed that etodolac treatment reduced the expression of COX-2 in both cell lines. To see if the 5-FU-resistance in UM-SCC-23F/R was related to the expression level of TS, we next determined the protein level of TS in both cell lines. Although expression of TS was not observed in UM-SCC-23 cells, UM-SCC-23F/R cells showed a high level of TS expression. This confirmed that 5-FU resistance was mediated by overexpression of TS. Surprisingly, etodolac treatment inhibited the expression of TS protein in UM-SCC-23F/R cells (Figure 3).

IL-1β treatment mimics 5-FU resistance. It is known that IL-1β enhances the COX-2 level in several types of cancer cell. Therefore, we next examined the sensitivity to 5-FU with IL-1β treatment (IL-1β at 1 ng/ml) in UM-SCC-23 and UM-SCC-23F/R cells. Both cell lines showed significant resistance to 5-FU in the presence of IL-1β (Figure 4A). 5-FU and etodolac combination treatment, even in the presence of IL-1β, showed that etodolac significantly increased sensitivity to 5-FU (Figure 4B, Table II).

Etodolac treatment down-regulates COX-2 and TS expression levels in the presence of IL-1β. In UM-SCC-23F/R cells, COX-2 expression was not increased upon IL-1β treatment; however, etodolac treatment reduced COX-2 protein expression

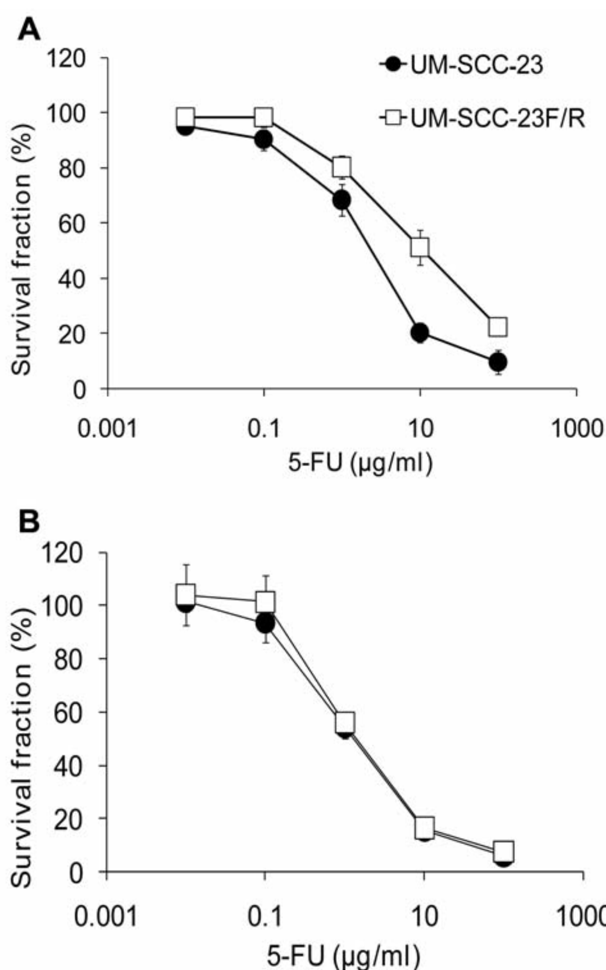

Figure 4. *A: Sensitivity of UM-SCC-23 and UM-SCC-23F/R cells treated with 5-FU in the presence of IL-1β. Each data point is the mean of three independent experiments. Error bars show standard deviations. B: Sensitivities of UM-SCC-23 and UM-SCC-23F/R cells treated with 5-FU and etodolac in the presence of IL-1β. Each data point is the mean of three independent experiments. Error bars show standard deviations. *$p<0.05$, t-test.*

in spite of the presence of IL-1β. In UM-SCC-23F/R cells, TS expression was increased after IL-1β treatment. However, etodolac inhibited the expression of TS even in the presence of IL-1β (Figure 5). These results suggest that etodolac treatment causes down-regulation of COX-2 and TS protein despite the presence of IL-1β in the culture medium.

Discussion

This study demonstrates a novel inhibitory effect of etodolac on TS expression in HNSCC 5-FU-resistant cells. We isolated the 5-FU-resistant cell line, UM-SCC-23F/R, from 5-FU-sensitive UM-SCC-23 cells by treating cells with an

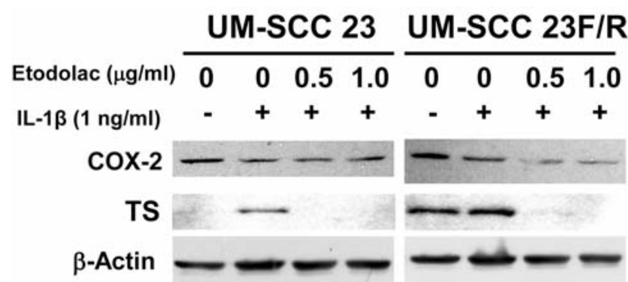

Figure 5. *Western blot analysis of cyclooxygenase-2 (COX-2) and thymidylate synthase (TS) expression on treatment with etodolac and interleukin-1β of UM-SCC-23 and UM-SCC-23F/R cells.*

increasing amount of 5-FU. We also demonstrated a sensitizing effect of the selective COX-2 inhibitor, etodolac, towards the cytotoxic action of 5-FU in 5-FU-resistant cells. This synergistic effect seems to be achieved *via* the inhibition of TS expression. Although we have not determined the effect of combination treatment of 5-FU and etodolac on TS expression, etodolac may overcome the effect of 5-FU on induction of TS protein because etodolac abolished the up-regulation of TS in 5-FU-resistant cells. Further investigation is required to determine the effect of combination treatment on TS expression.

5-FU-resistant mechanisms have not been clearly elucidated yet. Several studies demonstrate that the sensitivity to 5-FU is closely related to TS activity (16, 17). High level of TS protein expression is also associated with 5-FU resistance (18). In our study, expression of TS was not seen in UM-SCC-23 cells, but a high level of TS expression was observed in UM-SCC-23F/R cells. This finding indicates that cells have higher TS expression in the process of their acquisition of a 5-FU-resistant phenotype. Higher TS protein expression leads to cells becoming viable for DNA synthesis and survival despite 5-FU treatment (19).

The importance of TS enzyme inhibition was revealed from the use in a clinical setting of leucovorin (LV) or levofolinic acid (LFA) in combination with 5-FU. LV is intracellularly metabolized to the reduced folate 5,10-methylenetetrahydrofolate, which forms a complex with the 5-FU metabolite FdUMP and TS, resulting in a minimum level of TS activity. During the last decade, there were several clinical trials of LV and 5-FU for the treatment of some types of cancer, including colorectal and head and neck cancer. However, this combination regimen did not show improved overall survival of patients with advanced colorectal cancer, with one exceptional study. Therefore, more potent inhibitor compounds of TS were developed. These compounds, such as ZD1694, ZD9331, LY231514 and AG337, have been examined as novel cancer treatment combined with 5-FU under preclinical and clinical evaluation (20). Just recently, the FDA approved the use of FUSILEV® (levoleucovorin) in combination with 5-FU in the palliative treatment of patients with advanced metastatic colorectal cancer.

In this study, combination treatment of etodolac and 5-FU had a synergestic cytotoxic effect on UM-SCC-23 cells, Interestingly, this synergistic effect was determined at a dose of etodolac (0.5 μg/ml) which did not show any antitumor effect. Past studies reported that COX-2 inhibitors induced apoptosis (21), and combination of 5-FU and NSAIDs treatment showed synergistic cytotoxic effect in colorectal cancer (22). This should be completely unrelated to the mechanism of 5-FU action. Thus, further investigation is required for elucidation of this synergistic mechanism. As several reports have suggested that COX-2 contributes to progression (23), invasion (24), and angiogenesis (25) of HNSCC, this combination regimen may show further improvement in in vivo experiment and clinical settings in the treatment of HNSCC.

To the best of our knowledge, there is no report to demonstrate the inhibitory effect of COX-2 inhibitor on TS expression in cancer cells. In addition, we were able to successfully improve 5-FU sensitivity by COX-2 and TS inhibition using etodolac treatment in 5-FU-resistant tongue cancer cells. As TS plays an important role in 5-FU resistance, this study may contribute to the improvement of 5-FU resistance in the treatment of patients with HNSCC. Furthermore, etodolac demonstrates potential as a synergistic agent for use with 5-FU. Because etodolac has been widely used in conventional cancer pain treatment, the combination treatment of 5-FU with etodolac may have clinical applications in improving a patient's prognosis and quality of life.

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