Molecular mechanism underlying the synergistic interaction between trifluorothymidine and the epidermal growth factor receptor inhibitor erlotinib in human colorectal cancer cell lines

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The pyrimidine trifluorothymidine (TFT) inhibits thymidylate synthase (TS) and can be incorporated into the DNA. TFT, as part of TAS-102, is clinically evaluated in phase II studies as an oral chemotherapeutic agent. Erlotinib is a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) that is often deregulated in colorectal cancer. This study investigated molecular mechanisms underlying the cytotoxic actions of the combination of an EGFR-tyrosine kinase inhibitor with TFT in colorectal cancer cells Caco2, WiDr, Lov92, and Colo320. Drug interactions were examined by the sulforhodamine B assay and subsequent combination index (CI) analyses, cell cycle effects by FACS analysis of propidium iodide stained cells, Akt, MAPK and EGFR phosphorylation and expression levels by Western blotting and TS activity by the TS in situ assay. All combination schedules were synergistic in wt-EGFR expressing (but with mutated downstream pathways) WiDr and Lov92 (CI 0.4–0.8) and very synergistic in Caco2 cells (with wt-EGFR and functional downstream pathways; CI 0.1–0.3), but in EGFR-lacking Colo320 cells, no additional activity was found (CI 1.0–1.2). Synergism was mostly related to the induction of cell cycle arrest and an erlotinib-mediated inhibition of the pro-survival signaling through Akt and MAPK that was activated (phosphorylated) by TFT. Erlotinib inhibited TS activity in EGFR-expressing cell lines, probably due to cell cycle arrest in the G1 phase. TS activity was slightly lower in the combinations, probably due to cell cycle interference. Taken together, the combination of erlotinib with TFT seems to present a potential strategy in the field of molecular therapeutics. (Cancer Sci 2010; 101: 440–447)

Colorectal cancer is the third leading cause of cancer death worldwide. The standard therapy consists of 5FU and leucovorin, which is usually combined with either the topoisomerase I inhibitor irinotecan or the novel platinum analog oxaliplatin.(1) Addition of the latter compounds substantially increased the median survival. Further improvement of therapy can be expected from drugs bypassing 5FU resistance. Therefore 5FU prodrugs are currently being investigated, including S-1, UFT and capcitabine.(2) Recently, we and others showed that another fluoropyrimidine, TFT, might be more effective in CRC cells to overcome (acquired) 5FU resistance. Therefore 5FU prodrugs are currently being investigated, including S-1, UFT and capcitabine. The pyrimidine trifluorothymidine (TFT) inhibits thymidylate synthase (TS) and can be incorporated into the DNA. TFT, as part of TAS-102, is clinically evaluated in phase II studies as an oral chemotherapeutic agent. Erlotinib is a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) that is often deregulated in colorectal cancer. This study investigated molecular mechanisms underlying the cytotoxic actions of the combination of an EGFR-tyrosine kinase inhibitor with TFT in colorectal cancer cells Caco2, WiDr, Lov92, and Colo320. Drug interactions were examined by the sulforhodamine B assay and subsequent combination index (CI) analyses, cell cycle effects by FACS analysis of propidium iodide stained cells, Akt, MAPK and EGFR phosphorylation and expression levels by Western blotting and TS activity by the TS in situ assay. All combination schedules were synergistic in wt-EGFR expressing (but with mutated downstream pathways) WiDr and Lov92 (CI 0.4–0.8) and very synergistic in Caco2 cells (with wt-EGFR and functional downstream pathways; CI 0.1–0.3), but in EGFR-lacking Colo320 cells, no additional activity was found (CI 1.0–1.2). Synergism was mostly related to the induction of cell cycle arrest and an erlotinib-mediated inhibition of the pro-survival signaling through Akt and MAPK that was activated (phosphorylated) by TFT. Erlotinib inhibited TS activity in EGFR-expressing cell lines, probably due to cell cycle arrest in the G1 phase. TS activity was slightly lower in the combinations, probably due to cell cycle interference. Taken together, the combination of erlotinib with TFT seems to present a potential strategy in the field of molecular therapeutics. (Cancer Sci 2010; 101: 440–447)

Materials and Methods

Cell lines and chemicals. Human colon carcinoma cell lines WiDr, Colo320, Lov92 and Caco2 were cultured as monolayers in DMEM supplemented with 10% heat-inactivated FCS and 20 mM HEPES. Cells were maintained in a humidified 5% CO2 atmosphere at 37°C. TFT was provided by Taiho Pharmaceutical Co. (Tokushima, Japan).

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**Table 1. Characteristics of colorectal cancer cell lines used in this analysis**

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<tr>
<th></th>
<th>EGFR</th>
<th>p53</th>
<th>Braf</th>
<th>k-Ras</th>
<th>PTEN</th>
<th>IC$_{50}$ erlotinib (µM)</th>
<th>IC$_{50}$ TFT (µM)</th>
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<tbody>
<tr>
<td>WiDR</td>
<td>w.t.</td>
<td>mut</td>
<td>mut</td>
<td>w.t.</td>
<td>w.t.</td>
<td>8.2 ± 0.8</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Lovo92</td>
<td>w.t.</td>
<td>mut</td>
<td>mut</td>
<td>w.t.</td>
<td>w.t.</td>
<td>4.4 ± 1.3</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Colo320</td>
<td>w.t.</td>
<td>w.t.</td>
<td>mut</td>
<td>mut</td>
<td>w.t.</td>
<td>3.2 ± 0.3</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Caco2</td>
<td>w.t.</td>
<td>w.t.</td>
<td>w.t.</td>
<td>w.t.</td>
<td>w.t.</td>
<td>0.3 ± 0.1</td>
<td>25 ± 2.0</td>
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†Colo320 is w.t. p53, but lacks p21 (see Fig. 1) and therefore behaves as a mutant. EGFR, epidermal growth factor receptor; mut, mutant; TFT, trifluorothymidine; w.t., wild type.
line that is very sensitive to erlotinib (Fig. 1) due to a wild-type EGFR expression and with no reported mutations in the genes of p53, Braf, k-Ras, or PTEN. In addition, EGFR expression levels were higher in Caco2 than in WiDR cells.\textsuperscript{21} Caco2 cells were sensitive to erlotinib with an IC_{50} value in the nM range (Table 1; Fig. 1). In these cells, all three combination schedules were highly synergistic (Fig. 1).

**Effects on cell cycle distribution.** To determine whether the synergistic actions were related to specific cell cycle effects, \textsuperscript{39}FACS analysis of PI stained cells was carried out using IC_{50} concentrations (Table 1; Fig. 3; Fig. S1, Supporting Information). TFT alone induced predominantly a G2/M-phase arrest, although this was cell line- and time-dependent. In WiDR and Lovo92, erlotinib alone increased cells in the G1-phase, which was not time-dependent. In contrast, in Colo320 cells, erlotinib caused no significant changes in the cell cycle distribution. The simultaneous exposure increased WiDR cells in the G1 phase to some extent. When preincubated with erlotinib, the combined treatment resulted in an increased S phase fraction. In Lovo92 cells, all drug combinations induced G1-phase arrest. In addition, in both WiDR and Lovo92 cells, the cell cycle distribution seen after combined drug treatments is more comparable to that of erlotinib alone than to that of TFT alone. When Colo320 cells were preincubated with erlotinib prior to addition of the combination, cells mostly accumulated in the S phase, whereas the other two combinations arrested cells mainly in the G2/M phase, comparable to TFT alone. Because of the different cell cycle distribution in the pre-erlotinib schedule, erlotinib may have off-target effects. In Colo320 cells the combinations were not synergistic. These different effects on cell cycle distribution between the various cell lines indicate that the interactions between the drugs are cell cycle mediated.

**Effects on cell death induction.** To determine whether the combinations resulted in an increase in cell death induction, we analyzed the sub-G1-fraction of PI stained cells. In order to determine apoptosis we also determined caspase activation, which might not be seen in the sub-G1. The pattern of sub-G1 accumulation agreed with caspase activation (data not shown). In all three cell lines, TFT induced cell death in a time-dependent manner (Fig. 4). Erlotinib did not induce cell death directly. In WiDR, cell death was induced more strongly by the combinations compared to the control, although the combinations did not have a higher cell death than induced by 72 h TFT alone. In Lovo92 and Colo320 cells, cell death induced by the combination where TFT was given first was significantly higher than control levels, although lower than induced by TFT alone. As the combinations in EGFR expressing cells were synergistic, this may indicate that the combinations act by the induction of cell cycle arrest rather than cell death.

**Thymidylate synthase activity.** Thymidylate synthase is an important cell cycle enzyme that plays a limiting role in de novo pyrimidine deoxynucleotide synthesis. As the cell cycle effects seem to be important for the synergistic action of the combinations, and TS is one of the targets of TFT, we determined TS inhibition in intact cells treated with TFT and erlotinib (Table 2). Based on previous time-course experiments with TS inhibitors in these cell lines, we chose 24 h to measure the inhibition.\textsuperscript{22} TFT markedly inhibited TS activity in all three cell lines. Erlotinib alone also inhibited TS activity in EGFR expressing WiDR and Lovo92 cells. After a simultaneous combination, TS was inhibited to a larger extent than by TFT alone. This level of inhibition was almost similar to the expected level of inhibition, although the decrease was not significant (Table 2). The effect of erlotinib alone on TS might be related to a cell cycle-dependent activity of TS.\textsuperscript{15,23}

**Effects on EGFR, MAPK and Akt phosphorylation.** Erlotinib inhibits EGFR phosphorylation and thereby its downstream targets, Akt and MAPK. To determine whether cell cycle arrest was related to inhibition of these important cell growth signaling pathways, Western blot analysis was carried out. Lovo92 strongly expressed EGFR, WiDR had a moderate EGFR expression, but Colo320 did not express EGFR (Fig. 2).
Combinations of drug stimulation with EGF. EGFR was not detectable and could not be determined whether erlotinib can inhibit EGFR, EGF related to cell cycle effects, which in turn are related to the inhibition of the pro-survival signals that are induced by TFT. Overall, the inhibition of cell growth can be shown), we also investigated the effects of the combination after 24 h (Fig. 6). Surprisingly, in WiDR and Lovo92 cells, after 48 h of TFT exposure phosphorylated EGFR increased, which was reduced after 72 h. In both WiDR and Lovo92 cells TFT increased phosphorylation levels of MAPK and Akt time-dependently. Erlotinib barely affected Akt phosphorylation, but in WiDR cells, phosphorylated Akt increased after 24 h, which reduced in time.

In WiDR and Lovo92, addition of erlotinib prevented the pro-survival signalling that was induced by TFT, even to lower levels than the control phosphorylation levels. Although after the sequential combination where TFT was given first, Akt and MAPK were still activated, this was reduced to control levels. In Colo320 cells, which did not express detectable EGFR or phosphorylated MAPK, no changes in expression and phosphorylation status of histone H2A.X, one of the first events in DNA damage response. Cells exposed to the drugs or drug combinations induced a less than additive effect was seen. In Colo320 cells, this DNA damage increased significantly (up to 55-fold) following subsequent 48 h of culture in drug-free medium. In Colo320 cells this accumulation did not increase, but may explain the observed cell cycle changes after exposure to the combination.

As effects on phosphorylation are direct, the phosphorylation status of these proteins was determined after 2 h exposure of the drugs and simultaneous drug combination (Fig. 5). Moreover, to determine whether erlotinib can effectively inhibit EGFR, EGF was added to the cultures 5 min prior to cell lysis. In this way, it can be determined whether the EGFR signalling pathway was inhibited. In WiDR and Lovo92 cells, EGFR, Akt, and MAPK phosphorylation increased after EGF stimulation. TFT slightly increased the phosphorylation levels of MAPK. Erlotinib alone increased EGFR phosphorylation, but prevented the stimulation by EGF. Erlotinib clearly prevented activation of the downstream kinase Akt to a lesser extent that of MAPK. In the combination, phosphorylation of EGFR and Akt were inhibited almost completely, whereas the stimulation of MAPK by EGF and TFT was completely prevented. In Colo320 cells, EGFR, Akt, and MAPK phosphorylation levels did not change after stimulation with EGF. EGFR was not detectable and could not be upregulated to a detectable level by any of the tested drugs or drug combinations. Akt phosphorylation was slightly decreased after TFT alone. These results indicate that erlotinib can inhibit EGFR signalling in cells with an active EGFR, but has no effect on downstream signalling in cells with low or absent EGFR expression.

In order to investigate long-term effects, and the effects in the sequential combinations, which cannot be determined after 2 h and in which EGF addition does not play a role (data not shown), we also investigated the effects of the combination after 72 h (Fig. 6). DNA damage was monitored by determination of the level of DNA damage after 48 h of growth in drug-free medium. In Colo320 cells, this DNA damage increased significantly (up to 55-fold) following subsequent 48 h of culture in drug-free medium. In Colo320 cells this accumulation did not increase, but may explain the observed cell cycle changes after exposure to the combination.

**Effect on DNA damage.** Recent reports have indicated that EGFR inhibition might decrease DNA repair activity, therefore we determined the level of DNA damage after 24 h of drug incubation and whether DNA damage levels decreased (due to repair) after 48 h of growth in drug-free medium (Fig. 7). DNA damage was monitored by determination of the phosphorylation status of histone γ-H2A.X, one of the first events in DNA damage response. Cells exposed to the drugs or drug combinations induced γ-H2A.X phosphorylation, although for the combinations a less than additive effect was seen. In Lovo92 and WiDR cells, this DNA damage increased significantly (up to 55-fold) following subsequent 48 h of culture in drug-free medium. In Colo320 cells this accumulation did not increase, but may explain the observed cell cycle changes after exposure to the combination.

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**Fig. 3.** Cell cycle effects at IC50 concentrations of trifluorothymidine (TFT) (T), erlotinib (E), simultaneous combination (T + E), and the sequential combinations where cells were preincubated for 24 h with TFT (T → T + E) or E (E → T + E) followed by the T + E combination in WiDR, Lovo92 and Colo320 colorectal cancer cells. Values represent means of at least four independent experiments ± SEM. *Significant differences between treated and control (C) (P < 0.05).

**Fig. 4.** Cell death induction (sub-G1) after exposure to IC50 concentrations of trifluorothymidine (TFT) (T), erlotinib (E), or the IC50-ratio based combinations T + E, T → T + E (preincubated for 24 h with TFT, followed by T + E combination), or E → T + E (preincubated for 24 h with E, followed by T+E combination in WiDR, Lovo92, and Colo320 colorectal cancer cells. Values represent means of at least four independent experiments ± SEM. *Significant differences between treated and control (C) (P < 0.05).
Thymidylate synthase in situ activity in WiDR, Lovo92 and Colo320 colorectal cancer cells

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<tr>
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<th>TFT</th>
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<th>Combination</th>
<th>Expected</th>
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<tr>
<td>WiDR</td>
<td>2.1 ± 0.6</td>
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<td>Lovo92</td>
<td>33.6 ± 2.2</td>
<td>64.3 ± 10.2</td>
<td>24.3 ± 4.8</td>
<td>21.6</td>
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<td>Colo320</td>
<td>20.4 ± 2.2</td>
<td>101.5 ± 7.2</td>
<td>13.2 ± 3.4</td>
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Thymidylate synthase in situ activity after 24 h exposure to IC50 concentrations of either trifluorothymidine (TFT), erlotinib, or the simultaneous combination. Values represent the percentage of untreated control cells and are expressed as means of at least three independent experiments ± SEM. Expected values are calculated by multiplying the percentage inhibition by erlotinib by the percentage activity of TFT alone. When the value is lower than the expected value, this means that thymidylate synthase inhibition is synergistic.

Fig. 5. Western blot of expression levels of intracellular kinases in colorectal cancer cells after 2 h exposure to trifluorothymidine (TFT) alone, erlotinib (E) alone, or in simultaneous combination (T + E) with or without epidermal growth factor (EGF) to stimulate EGFR-related signaling. The blots are representative of two or three independent experiments. Colo320 cells did not show any detectable expression of phosphorylated (p-)EGFR or phosphorylated MAPK. E → T + E, cells preincubated with E, followed by T + E combination; T → T + E, cells preincubated with TFT, followed by T + E combination.

WiDR

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<td>p44/42 MAPK</td>
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<td>β-actin</td>
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Lovo92

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Colo320

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<td></td>
<td>β-actin</td>
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Fig. 6. Western blot of expression levels of intracellular kinases after 24, 48, and 72 h exposure to trifluorothymidine (TFT) alone, erlotinib (E) alone, or in combination schedules (72 h). The blots are representative of three independent experiments. Colo320 cells did not express detectable epidermal growth factor receptor (EGFR), phosphorylated (p-)EGFR, or phosphorylated MAPK (Fig. 5), so blots have not been included in this figure. C, control; E → T + E, cells preincubated with E, followed by T + E combination; T → T + E, cells preincubated with TFT, followed by T + E combination; T + E, simultaneous combination of TFT and E.

Discussion

This study shows that a combination of TFT with erlotinib is synergistic in EGFR expressing colon cancer cells, also in the presence of a k-Ras mutation. This sensitization of moderately sensitive cells to erlotinib indicates the potential for evaluating the combination of TFT with EGFR-TKIs. Ongoing clinical studies are confirming the promising therapeutic activity of second-generation EGFR-TKIs and multitargeted TKIs in several tumor types.(26,27) The combination of TFT with erlotinib was strongly synergistic in Caco-2 cells with a functional (e.g. wild-type) EGFR signaling pathway, and the combination was synergistic in WiDR and Lovo92 cells that were moderately sensitive to erlotinib with mutations in the downstream EGFR pathway. The combination was not synergistic in cells that did not express EGFR. This underlines the importance of an active EGFR signaling pathway in the synergistic interaction. The mechanism underlying the synergistic interaction was probably due to inhibition of the downstream EGFR pro-survival signaling pathway and the induction of DNA damage.

Synergistic actions with EGFR-TKIs have previously been reported with cytotoxic agents such as 5FU and TS inhibitors (pemetrexed) and irinotecan, and with radiation.(15,28–30) These cytotoxic agents and radiation could increase the phosphorylation level of EGFR, which possibly reflects the activation of pro-survival signaling. This effect was blocked by addition of
EGFR-TKIs, explaining the synergistic activities. 5FU or pemetrexed-induced activation of EGFR phosphorylation was prevented by gefitinib and erlotinib.\(^{(15,31)}\) In our study, such a synergistic interaction was only found for cells that constitutively expressed both EGFR and activated the pro-survival signaling after exposure to TTF. TTF alone activated EGFR phosphorylation levels, and highly increased the phosphorylation levels of the pro-survival kinases MAPK and Akt. This pro-survival signaling could be inhibited by the addition of erlotinib, possibly explaining the synergistic interaction. In EGFR lacking Colo320 cells, erlotinib could inhibit cell growth and no synergism was found. In these cells TTF hardly activated pro-survival signaling and erlotinib did not have any effect on the phosphorylation levels of the tested intracellular kinases or on the cell cycle. Therefore, it is likely that the mechanism of erlotinib in these cells do not involve changes in intracellular signaling in the examined routes.

All three cell lines were moderately sensitive to erlotinib. In NSCLC and CRC, mutations in \(k\)-Ras and \(BRAF\) are potential biomarkers for erlotinib sensitivity. Mutations in these genes can cause constitutive activation of MAPK. The low EGFR expression in one cell line and \(k\)-Ras mutations may explain the low sensitivity to erlotinib. However, independent of the mutations in \(k\)-Ras, downstream signaling of Akt and MAPK may be significantly inhibited in our cell lines. In addition, EGFR status is known not to be a predictive factor for responses to cetuximab, as responses were reported in patients with EGFR-negative tumors.\(^{(32,33)}\) In addition, erlotinib has shown to have some off-target effects in leukemia cells, in which the JAK2/STAT5 pathway was inhibited.\(^{(34)}\) and which were likely to be comparable to the off-target effects of gefitinib.\(^{(35)}\) In the latter studies, differentiation and cell cycle blockade induced by EGFR antagonists exerting off-target effects on Acute Myeloid Leukemia (AML) cells were not automatically linked to an apoptotic response.\(^{(34,35)}\) Our data indicate that erlotinib also has off-target effects in colon cancer cells. Ongoing clinical studies, in which the responses to the new-generation EGFR-TKIs are evaluated, appear to be promising. In this respect, based on our current data, the inhibition of pro-survival signaling by EGFR-TKIs can enhance the efficacy of the chemotherapeutic agent and may be worthwhile to test in a clinical setting.

Recent studies reported that EGFR-TKIs can decrease the expression levels and activity of TS in NSCLC, breast, and colorectal cancer cells and could be related to synergism. As erlotinib did not inhibit TS itself in cell-free extracts,\(^{(15)}\) the decrease in TS can be explained by the \(G_1/G_0\) cell cycle arrest that was induced by the EGFR-TKI. TS is an enzyme that is only active in the \(S\) phase of the cell cycle. TS activity was decreased in the combinations, compared to TTF alone, which is possibly related to an increased inhibition of TS and the induced cell cycle arrest.\(^{(15)}\)

Epidermal growth factor receptor inhibitors have been shown to stimulate the induction of apoptosis in various cell types.\(^{(15,31)}\) However, in our colon cancer cell line panel, erlotinib itself did not induce cell death, as shown by the sub-\(G_1\) analysis. The lack of cell death is supported by the absence of caspase activation following erlotinib treatment. Erlotinib seems to act more on the cell cycle, arresting cells in the \(G_0/G_1\) phase, which can be mediated by inhibition of pro-survival signaling. As cell death was not involved in the synergistic actions, it is expected that the action of the combinations is more related to an induction of cell cycle arrest, possibly mediated by the induction of DNA damage. Previously it was reported that cell cycle modulation is important for the efficacy of the combination of EGFR-TKIs with cytotoxic agents.\(^{(15,37)}\) Cellular damage induced by chemotherapeutic compounds can convert EGFR ligands from growth factors into survival factors for cancer cells that express functional EGFR.\(^{(38)}\) In this context, the blockade of EGFR signaling by EGFR-TKI could prevent repair of cellular damage induced by cytotoxic drugs.

Inhibition of EGFR signaling has previously been shown to reduce DNA damage repair.\(^{(39)}\) TTF is a known inducer of DNA damage,\(^{(34,36)}\) therefore the combined treatment with erlotinib might cause persistent DNA damage induction, for example, TTF-induced DNA damage cannot be repaired due to inhibition of the DNA repair mechanisms by erlotinib.\(^{(39)}\) This notion is further strengthened by the finding that persistent DNA damage was only observed in EGFR expressing cells. This may also explain why erlotinib alone induced DNA damage, as it also affects basal repair levels. Thus, the synergism found between TTF and erlotinib might be explained by a diminished ability to repair TTF-induced DNA damage lesions.

Thymidine phosphorylase plays a role in apoptosis, cell growth, and angiogenesis.\(^{(40)}\) Moreover, TP might be induced after exposure to EGFR-TKIs and cytotoxic agents, which has been related to chemoresistance.\(^{(23,36)}\) \(\text{TAS}-102\) consists of TTF combined with thymidine phosphorylase inhibitor.\(^{(3)}\) This may be an additional advantage of a clinical combination of \(\text{TAS}-102\) with erlotinib. Further investigation is required to elucidate the role of TP in enhancement of responses to EGFR-TKI, especially \(\text{in vivo}\). In conclusion, the combination of TTF with EGFR-TKI was synergistic, which was dependent on EGFR expression and probably mediated by cell cycle deregulation and not immediate cell death. Although further research is needed to fully elucidate this promising combination in CRC therapy, the concept of combined molecular targeting of EGFR and TTF seems to present a potential strategy in the field of molecular therapeutics.

**Acknowledgment**

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Disclosure Statement
G. J. Peters received a research grant from Taiho Pharmaceutical Co. Ltd; M. Fukushima is an employee of Taiho Pharmaceutical Co. Ltd.

Abbreviations

SFU 5-fluorouracil
CI combination index

References

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Example of histograms of cell cycle distribution by FACS analysis of WiDR colorectal cancer cells after 72 h exposure to trifluorothymidine (TFT) (T) alone, erlotinib (E) alone, or in combination. E → T + E, cells preincubated with E, followed by T + E combination; T → T + E, cells preincubated with trifluorothymidine (TFT) (T), followed by T + E combination; T + E, simultaneous combination of TFT and E.

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