

# Chapter 6

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**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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Irene V. Bijnsdorp<sup>1</sup>

Frank A. Kruyt<sup>1,2</sup>

Masakazu Fukushima<sup>3</sup>

Kees Smid<sup>1</sup>

Shanti Gokoel<sup>1</sup>

Godefridus J. Peters<sup>1</sup>

1. Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

2. Department of Medical Oncology, University Medical Center Groningen, Groningen, The Netherlands

3. Tokushima Research Center, Taiho Pharmaceutical Co., Ltd., Tokushima, Japan

### ABSTRACT

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-TKI with TFT in colorectal cancer cells Caco2, WiDR, Lovo92 and Colo320. Drug interactions were examined by the SRB-assay and subsequent combination index (CI) analyses, cell cycle effects by FACS analysis of PI 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 Lovo92 (CI: 0.4-0.8) and very synergistic in Caco2 cells (with wt-EGFR and functional downstream pathways; CI: 0.1-0.3), while 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 via Akt and MAPK that was activated (phosphorylated) by TFT. Erlotinib inhibited TS activity in EGFR-expressing cell lines, probably due to the cell cycle arrest in the G<sub>1</sub>-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.

### INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer death in the world. The standard therapy consist of 5-Fluorouracil (5-FU) and leucovorin (LV), which is usually combined with either the topoisomerase I inhibitor irinotecan (FOLFIRI) or the novel platinum analog oxaliplatin (FOLFOX)<sup>1</sup>. Addition of the latter compounds substantially increased the median survival. Further improvement of therapy can be expected from drugs bypassing 5-FU resistance. Therefore 5-FU prodrugs are currently investigated, including S-1, UFT and capecitabine<sup>2</sup>. Recently, we and others demonstrated that another fluoropyrimidine, TFT, might be more effective in CRC cells to overcome (acquired) 5-FU and/or FdUrd resistance<sup>3,4</sup>. TFT is part of the oral fluoropyrimidine formulation TAS-102 and can be activated to its phosphate derivatives. When monophosphorylated, TFT can inhibit thymidylate synthase (TS), thereby inhibiting the synthesis of new pyrimidines<sup>3</sup>. In its

triphosphate form, TFT can be incorporated into the DNA, causing DNA damage. Both events will lead to the induction of cell death. TAS-102 is currently tested in a phase II clinical trial against colon and gastric cancer<sup>3</sup>. TAS-102 is a promising novel formulation which also showed pronounced synergism with irinotecan and oxaliplatin<sup>5,6</sup>.

EGFR is overexpressed or deregulated in many human cancer types, including CRC<sup>7</sup>. A high EGFR expression level has been related to a poor prognosis<sup>8,9</sup>. Targeting EGFR may therefore be a rational approach to treat CRC patients. EGFR inhibitors are under investigation, including the tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib (Tarceva). Erlotinib is a highly potent reversible inhibitor of the tyrosine kinase domain of EGFR. Erlotinib is an oral compound that is active against a wide range of colon cancers *in vitro*<sup>10</sup>, but its clinical application is mostly limited to NSCLC in which it is active against patients with activating EGFR mutations and wild type k-Ras. Erlotinib as a monotherapy has also shown activity in metastatic CRC patients<sup>11</sup>, providing a basis for further studies in phase II in combination with chemotherapy. However, a proper analysis of the mechanism underlying the combination is essential. Although a combination of EGFR inhibition with chemotherapeutic regimens may enhance the anticancer response<sup>12,13</sup>, the interaction may be schedule and concentration dependent. The combination of the multitargeted TS inhibitor pemetrexed with erlotinib showed a sequence dependent synergistic interaction, e.g. when cells were pre-exposed to pemetrexed in NSCLC cell lines<sup>14,15</sup>. Various combination studies with erlotinib are currently under investigation in advanced and metastatic CRC, including with capecitabine and oxaliplatin, capecitabine and irinotecan, but also with bevacizumab and FOLFOX<sup>16,17,18</sup>.

The aim of our study was to investigate whether inhibition of EGFR could enhance the effect of TFT against CRC cells *in vitro*. We used erlotinib as a model-TKI and investigated schedule dependency in relation to the phosphorylation levels of EGFR as well as that of the intracellular kinases Akt and MAPK. Furthermore, we assessed the effect on cell cycle distribution, modulation of TS activity and the induction of DNA damage, to gain insight into the mechanism underlying the synergistic effect of combination therapy with TFT and an EGFR-TKI.

## MATERIALS AND METHODS

### *Cell lines and chemicals*

Human colon carcinoma cell lines WiDR, Colo320, Lovo92 and Caco2 were cultured as monolayers in DMEM supplemented with 10% heat inactivated FCS and 20 mM Hepes.

## Chapter 6

Cells were maintained in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. TFT was provided by Taiho pharmaceuticals Co. Ltd. (Tokushima, Japan).

### *Drug cytotoxicity assays*

Drug cytotoxicity was determined by the sulforhodamine B (SRB)-assay as described previously<sup>19</sup>. In 96-wells plates (Greiner Bio-One, Frickenhausen, Germany), 5000 cells/well were seeded in 100 µl medium. After 24 h, 100 µl drug containing medium was added to the wells. After 72 h drug exposure, cells were precipitated for 1 h at 4 °C with 25 µl 50% trichloroacetic acid (TCA) and colored with SRB (0.4% SRB in 1% Acetic acid (w/v)). The optical density was measured at 540 nm after reconstitution of the dye in 150 µl 10 mM Tris). IC<sub>50</sub> values were estimated from graphs and are given in means ± SEM. For combination experiments, a concentration range which was fitted according to Calcsyn (see below) was used with a fixed ratio based on the IC<sub>50</sub>. Cells were either exposed concurrently to both TFT and erlotinib for 72 h or 24 h pre-incubated with either TFT or erlotinib, followed by a 48 h exposure to the combination. The interaction was determined with the multiple drug effect method, in which a combination index (CI) was calculated with Calcsyn (Calcsyn Biosoft, Cambridge, UK) as described previously<sup>5</sup>. This method is distinct from other methods by the fact that both 'potency' and 'shape' of dose-effect curves of drugs and their combinations are taken into account. For calculation of the CI, only values above a fraction affected (FA) of 0.5 were used, equivalent to 50 - 100% growth inhibition. Per experiment, CI values at FA 0.5, 0.75 and 0.9 were averaged according to the guidelines<sup>5</sup> and the mean was used for comparison of separate experiments. A CI < 0.9 indicates synergism and > 1.1 antagonism.

### *FACS analysis of cell cycle*

Cell cycle analysis and cell death measurement were performed as described previously<sup>19</sup>. The time-course of cell cycle changes for various combinations with TFT was determined earlier, showing the most pronounced changes after 72 h. Therefore, we focused on 72 h treatment with IC<sub>50</sub> concentrations of the drugs and drug-combinations; this also enabled the cells to have completed enough cell cycles (at least 3 cycles) to determine statistically significant differences. In brief, 100000 cells were seeded in 6-wells plates. Cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 4 min at 1200 rpm. Subsequently, cells were stained with propidium iodide (PI; 0.1 mg/ml) in dark on ice. DNA content of the cells was analyzed by FACS (FACScan Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10000 events. Sub-G<sub>1</sub> peak was used as indication of cell death induction.

*TS in situ activity*

Inhibition of thymidylate synthase (TS) in intact cells was determined by measuring the conversion of [<sup>3</sup>H]-dCyd to <sup>3</sup>H<sub>2</sub>O, which is catalyzed by TS as described previously<sup>20</sup>. Briefly, 1x10<sup>6</sup> cells were seeded and incubated with the IC<sub>50</sub> concentrations of the different drugs and the simultaneous combination for 22 h. Subsequently, [<sup>3</sup>H]-dCyd (final concentration: 1 μM, specific activity 4.9 Ci/mmol) was added to each sample for 2 h at 37°C. Blanks consisted of culture medium only and as controls untreated cells were used. The reaction was stopped by adding TCA and unconverted [<sup>3</sup>H]-dCyd was removed by activated charcoal. After centrifugation, the supernatant was transferred to a liquid scintillation vial and counted.

*Western blotting*

Cells were seeded in T25 culture flasks at a density of 1.5x10<sup>6</sup> cells. After 24 h, cells were exposed to IC<sub>50</sub> concentrations of TFT or the various combination schedules (IC<sub>50</sub> concentration) as described in the drugs cytotoxicity assay. After treatment for the indicated time-points in the figures, cells were lysed in lysis buffer (10% glycerol, 5 mM EDTA, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM β-glycerophosphate, 1% Triton X-100, 0.04% protease inhibitor cocktail (PIC), 0.1% 1 M (NaVO<sub>3</sub>)) and centrifuged at 11000 g at 4°C for 10 min. Protein concentration in the supernatant was determined by performing a Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, the Netherlands). From each condition 30 μg of protein was separated on a 10-12% SDS-PAGE and electroblotted onto a PVDF membrane. Blots were blocked in 5% milk in TBST (0.15 M NaCl, 0.05% Tween-20, 10 mM Tris-HCl, pH 8.0) and subsequently incubated at 4 °C overnight (O/N) with the following antibodies: EGFR (#2232), phospho-EGFR (Tyr1068 #2234), Akt (#9272), phospho-Akt, (Ser473 #9271) p42/44 MAP Kinase (#9102), phospho-p42/44 MAP kinase (Thr202/Tyr204 #9101) (1:1000; Cell Signaling Technology, Danvers, USA), p53 (AB-2; Oncogene Research products, Cambridge, USA), p21 (SantaCruz, California, USA) or phospho-γ-H2Ax (Ser139 #05-636; 1:1000, Upstate, Billerica, MA, USA) and β-Actin (#A5441; 1:10,000; Sigma Aldrich Chemicals, Deisenhofer, Germany) served as loading control for protein amount. After the first antibody, secondary antibody was added and signal was detected using ECL or ECL-plus on hyperfilms (Amersham International, Chalfont St. Giles, UK).

## RESULTS

*Growth inhibition and combination analysis*

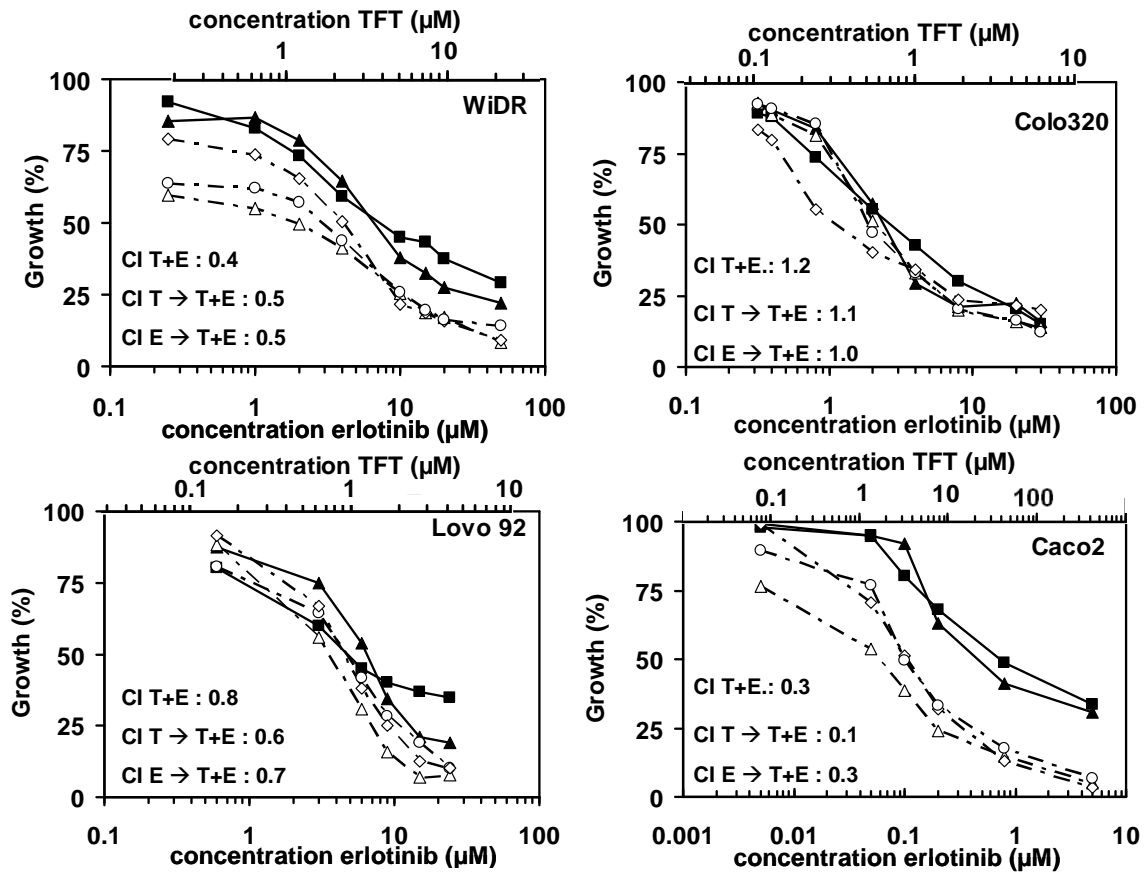
WiDR, Colo320 and Lovo92 showed comparable levels of sensitivity to TFT treatment as determined by the SRB-assay (Table 1; Figure 1). Cells were slightly more sensitive to TFT than to erlotinib. There was hardly a difference in sensitivity to erlotinib in the three cell lines, which were all relatively insensitive with  $IC_{50}$ s in the  $\mu$ M range. This is probably because they have a different mutation status for several kinases, including EGFR (Table 1). Furthermore, they have different expression levels of p53 and p21 (Figure 2). In WiDR cells, all combinations were synergistic. All combinations in Lovo92 cells were moderately synergistic, although at lower concentration ranges (growth inhibition <50%), the combinations were not synergistic (Figure 1). However, we do not consider this low concentration range of therapeutic interest. In Colo320 cells the tested combinations were not synergistic, although antagonism was not observed. In contrast to previous experiences with erlotinib combined with a TS inhibitor<sup>14,15</sup>, the order of administration of the combination schedule was not important to achieve synergism.

**Table 1 - Characteristics of the used cell line panel**

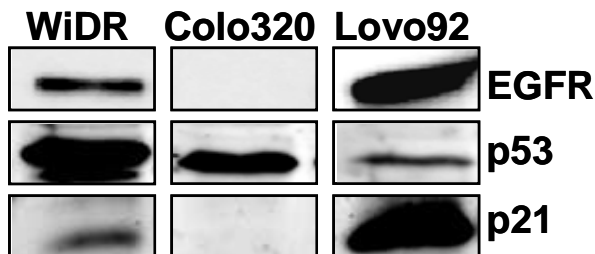
	EGFR	p53	Braf	k-Ras	PTEN	$IC_{50}$ erlotinib ( $\mu$ M)	$IC_{50}$ TFT ( $\mu$ M)
WiDR	w.t.	mut	mut	w.t.	w.t.	8.2 $\pm$ 0.8	3.2 $\pm$ 0.3
Lovo92	w.t.	w.t.	w.t.	mut	w.t.	4.4 $\pm$ 1.3	0.9 $\pm$ 0.1
Colo320	w.t.	w.t.*	w.t.	mut	w.t.	3.2 $\pm$ 0.3	0.6 $\pm$ 0.1
Caco2	w.t.	w.t.	w.t.	w.t.	w.t.	0.3 $\pm$ 0.1	25 $\pm$ 2.0

w.t. = wild type, mut = mutant. \* Colo320 is w.t. p53, but lacks p21 (see Fig. 1) and therefore behaves as a mutant.

In order to determine whether the sensitivity to erlotinib is important for synergism with TFT, we included Caco2, a cell line that is very sensitive to erlotinib (Figure 1) due to a wild-type EGFR expression and with no reported mutations in the genes of p53, Braf, k-Ras, PTEN. In addition, EGFR expression levels were higher in Caco2 than in WiDR cells<sup>21</sup>. Caco2 cells were sensitive to erlotinib with an  $IC_{50}$  value in the nM range (Table 1; Figure 1). In these cells, all three combination schedules were highly synergistic (Figure 1).



**Figure 1** - Growth inhibition curves of WiDR, Lovo92, Colo320 and Caco2 cells. Cells were exposed for 72h to TFT (T) (-■-; scale on upper part), erlotinib (E) (-▲-; scale on lower part), Simultaneous combination (T+E; -◇-), 24h pre-incubation with TFT followed by a 48 h combination (T → T+E; -Δ-) or a 24h pre-incubation with erlotinib followed by a 48 h combination (E → T+E; -○-). SEM was less than 20%. A mean combination index (CI) was calculated at fa of 0.5, 0.75 and 0.9 and values are indicated in the graphs for each combination. CI values represent means of at least 4 independent experiments and are given in the graphs for each combination with a SEM less than 20%.



**Figure 2** - Characterization of the cell lines by Western blot for EGFR, p53 and p21 expression levels in WiDR, Lovo92 and Colo320 cells.

## Chapter 6

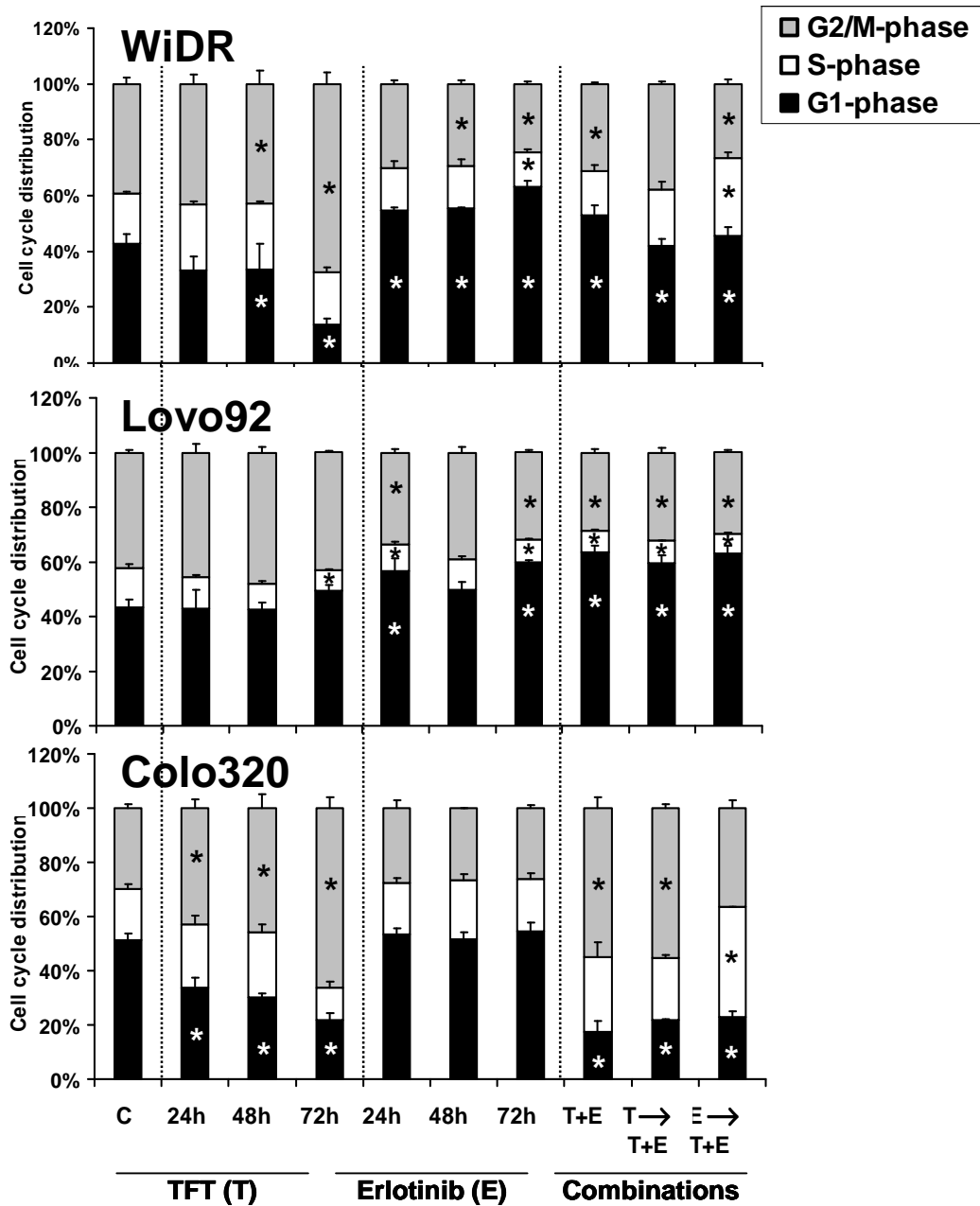
### *Effects on cell cycle distribution*

To determine whether the synergistic actions were related to specific cell cycle effects, FACS analysis of PI stained cells was performed, using  $IC_{50}$  concentrations (Table 1; Figure 3). TFT alone induced predominantly a  $G_2/M$ -phase arrest, although this was cell line and time dependent. In WiDR and Lovo92, erlotinib alone increased cells in the  $G_1$ -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  $G_1$ -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  $G_1$ -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, while the other two combinations arrested cells mainly in the  $G_2/M$ -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.

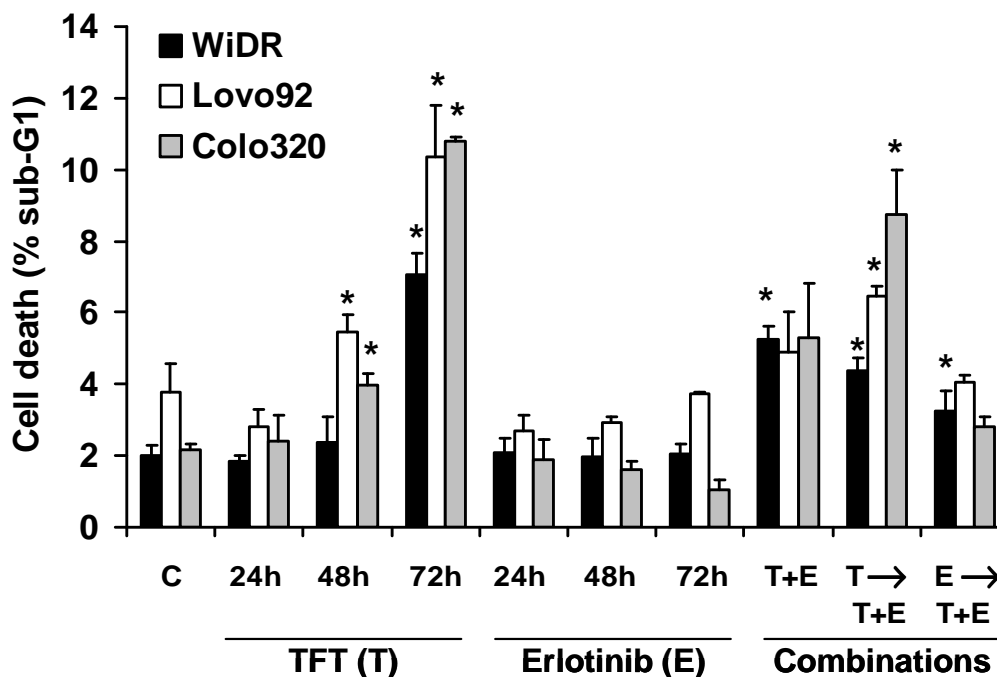
### *Effects on cell death induction*

To determine whether the combinations resulted in an increase in cell death induction, we analyzed the sub- $G_1$  fraction of PI stained cells. In order to determine apoptosis we also determined caspase activation, which might not be seen in the sub- $G_1$ . The pattern of sub- $G_1$  accumulation agreed with caspase activation (data not shown). In all three cell lines, TFT induced cell death in a time dependent manner (Figure 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. Since 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.





**Figure 3** - Cell cycle effects at IC<sub>50</sub> concentrations of TFT (T), erlotinib (E), the simultaneous combination (T+E), and the sequential combinations where cells were 24 h preincubated with TFT (T → T+E) or erlotinib (E → T+E) followed by the combination in WiDR, Lovo92 and Colo320 cells. Values represent means of at least 4 independent experiments ± SEM. Significant differences between treated and control are indicated with \* (p < 0.05).



**Figure 4** - Cell death induction (sub-G<sub>1</sub>) after exposure to IC<sub>50</sub> concentrations TFT (T), erlotinib (E) or the IC<sub>50</sub>-ratio based combinations T+E, T → T+E and E → T+E in WiDR, Lovo92 and Colo320 cells. Values represent means of at least 4 independent experiments ± SEM. Significant differences between treated and control are indicated with \* (p < 0.05).

#### *Thymidylate synthase activity*

TS is an important cell cycle enzyme that plays a limiting role in *de novo* pyrimidine deoxynucleotide synthesis. Since 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<sup>22</sup>. 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 may be related to a cell cycle dependent activity of TS<sup>15,23</sup>.

**Table 2 - TS *in situ* activity in WiDR, Lovo92 and Colo320 cells**

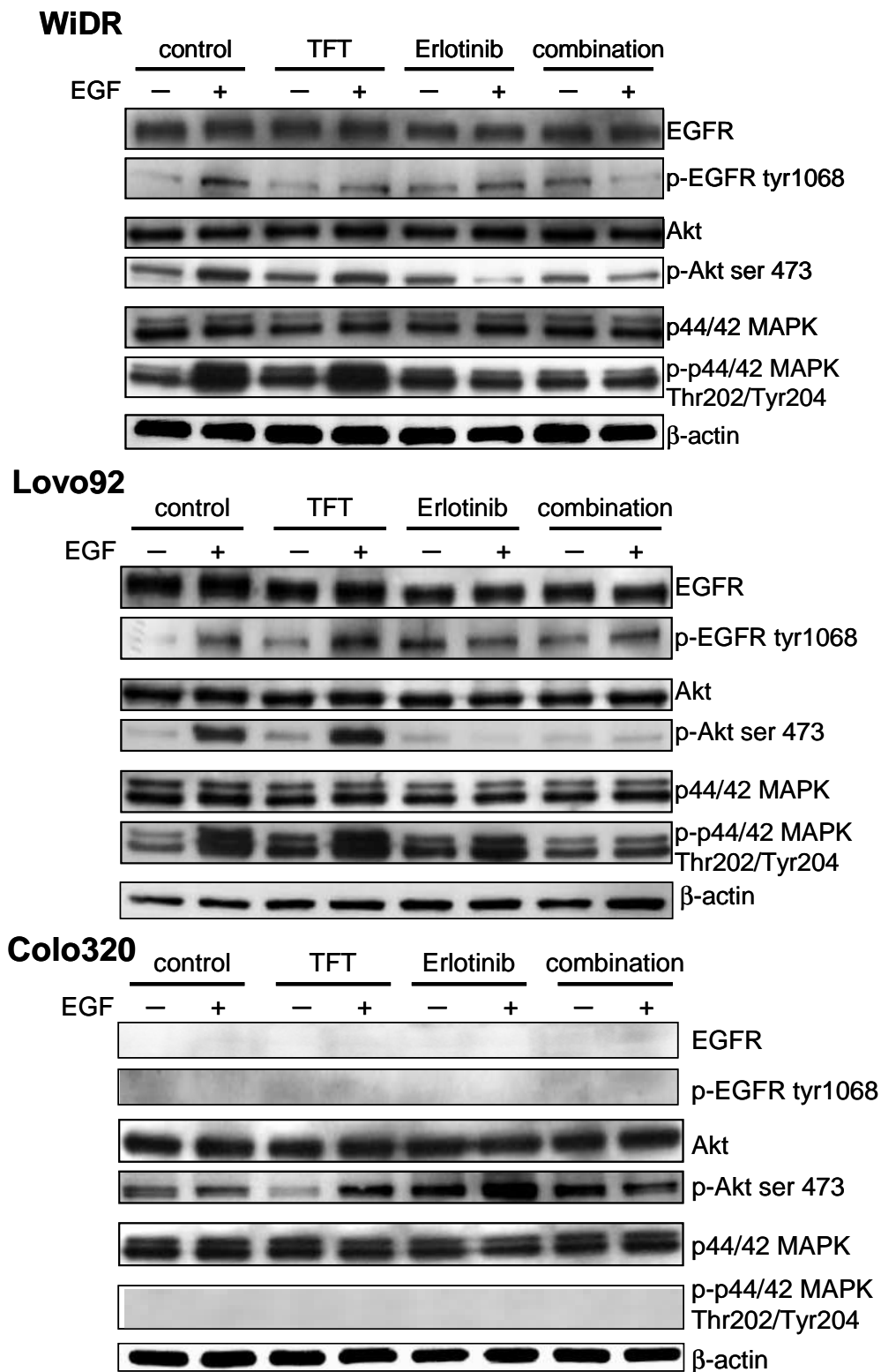
	TFT	Erlotinib	Combination	Expected
<b>WiDR</b>	2.1 ± 0.6	37.4 ± 4.8	0.8 ± 0.2	0.8
<b>Lovo92</b>	33.6 ± 2.2	64.3 ± 10.2	24.3 ± 4.8	21.6
<b>Colo320</b>	20.4 ± 2.2	101.5 ± 7.2	13.2 ± 3.4	20.7

TS *in situ* activity after 24 h exposure to IC<sub>50</sub> concentrations of either TFT, erlotinib or the simultaneous combination. Values represent % of untreated control cells and are expressed as means of at least 3 independent experiments ± SEM. Expected values are calculated by multiplying the % inhibition by erlotinib by that of % activity of TFT alone. When the value is lower than the expected value, this means that TS inhibition is synergistic.

#### *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 signalling pathways, Western blotting experiments were performed. Lovo92 strongly expressed EGFR, WiDR had a moderate EGFR expression, but Colo320 did not express EGFR (Figure 2).

Since effects on phosphorylation are direct, phosphorylation status of these proteins was determined after 2 h exposure of the drugs and simultaneous drug-combination (Figure 5). Moreover, to determine whether erlotinib can effectively inhibit EGFR, EGF was added to the cultures 5 minutes 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 and to a lesser extent that of MAPK. In the combination, phosphorylation of EGFR and Akt were inhibited almost completely while the stimulation of MAPK by EGF and TFT was completely prevented. In Colo320 cells, EGFR, Akt or 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 signaling in cells with low or absent EGFR expression.



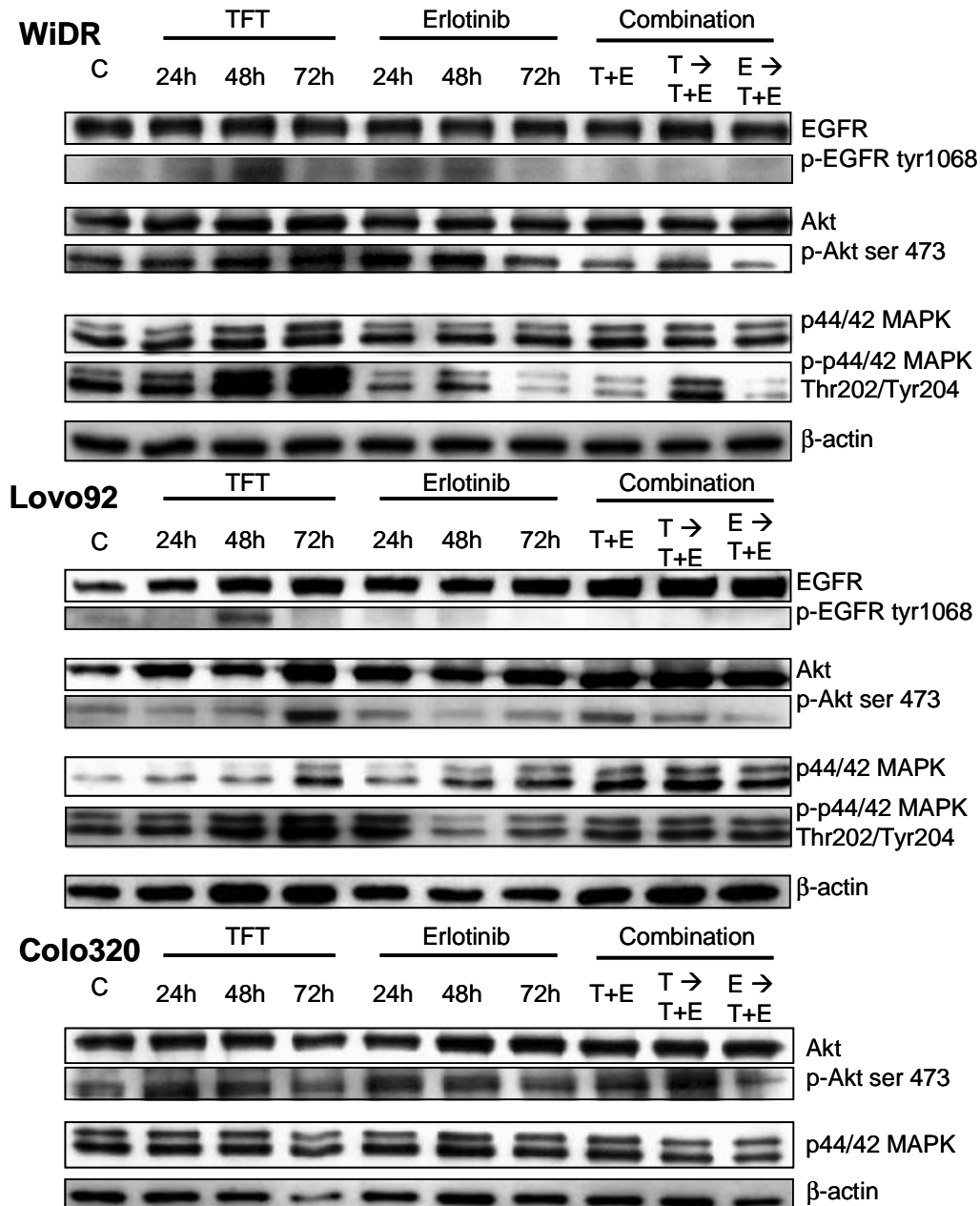
**Figure 5** - Western blot of expression levels of intracellular kinases after 2 h exposure to TFT or erlotinib alone and the simultaneous combination with and without EGF to stimulate EGFR related signaling. The blots are representative of 2-3 independent experiments. Colo320 cells did not show any detectable expression of (phosphorylated)-EGFR and phosphorylated MAPK.

In order to investigate the long term effects, and the effects in the sequential combinations, which can not 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 (Figure 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 hardly affected Akt-phosphorylation, but in WiDR phosphorylated-Akt increased after 24h, which reduced in time.

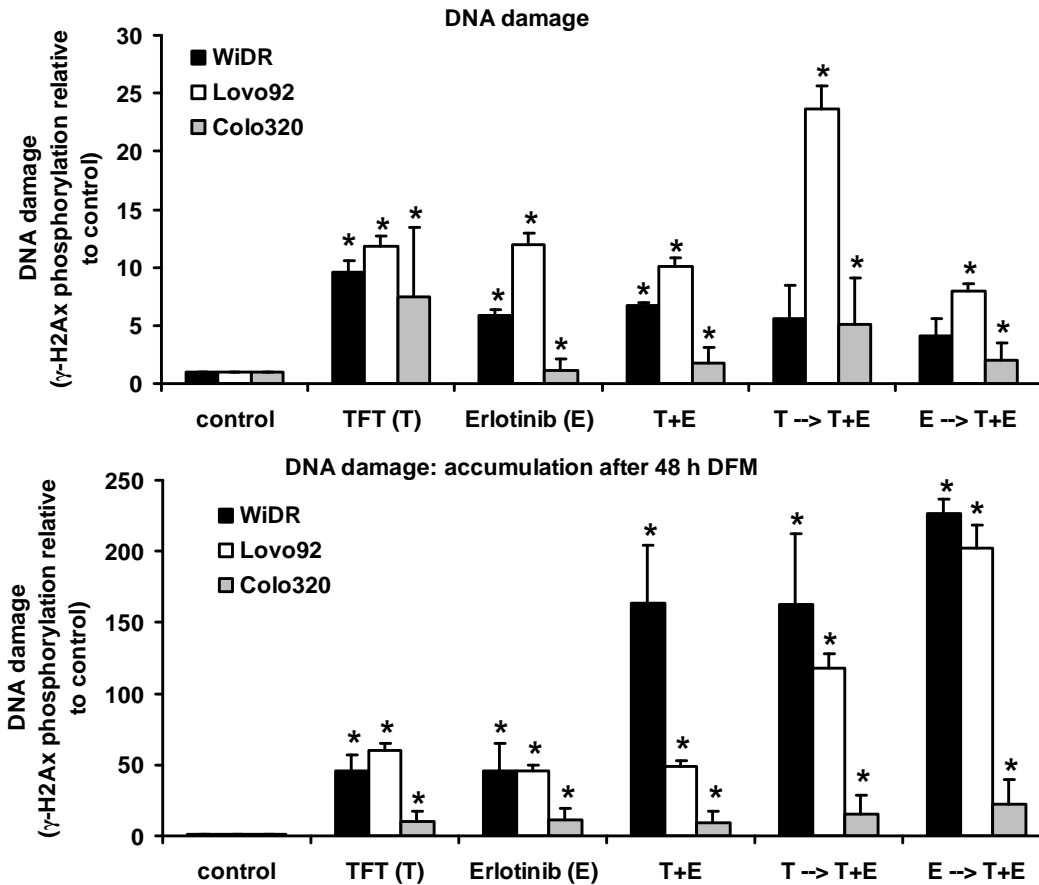
In WiDR and Lovo92, addition of erlotinib prevented the pro-survival signalling that was induced by TFT, even to lower levels than the control-phosphorylation levels. Although after the sequential combination where TFT was given first, Akt and MAPK were still activated, this was reduced to control levels. In Colo320, which did not express detectable EGFR or phosphorylated MAPK, no changes in expression and/or phosphorylation levels were observed after all different drugs and drug-combinations. Overall, the inhibition of cell growth can be related to cell cycle effects, which in turn are related to the inhibition of the pro-survival signals that are induced by TFT.

#### *Effect on DNA damage*

Since recent reports indicated that EGFR inhibition may decrease DNA repair activity<sup>24,25</sup>, we determined the level of DNA damage after 72 h drug incubation and whether DNA damage levels decreased (due to repair) after 48 h growth in drug free medium (Figure 7). DNA damage was monitored by determination of the phosphorylation status of histone  $\gamma$ -H2Ax, one of the first events in DNA damage response. Cells exposed to the drugs or drug-combinations induced  $\gamma$ -H2Ax phosphorylation, although for the combinations a less than additive effect was seen. In Lovo92 and WiDR cells, this DNA damage increased tremendously (up to 55 fold) following subsequent 48 h 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.



**Figure 6** - Western blot of expression levels of intracellular kinases after 24, 48 and 72 h exposure to TFT or erlotinib alone and the three combination schedules (72 h). The blots are representative of 3 independent experiments. Since Colo320 cells did not express detectable EGFR, phosphorylated EGFR and phosphorylated MAPK (Figure 5), blots are not included in the figure.



**Figure 7** - Phosphorylation levels of  $\gamma$ -H2Ax as a marker for DNA damage induction as determined by Western blotting after 72 h incubation with IC<sub>50</sub> concentrations and DNA damage accumulation after 72 h drug exposure and subsequently 48 h recovery in drug free medium. Values represent means corrected for control phosphorylation levels (set to 1)  $\pm$  SEM. Significant differences between treated and control are indicated with \* ( $p < 0.05$ ).

## DISCUSSION

This study demonstrates 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 demonstrates the potential for evaluating the combination of TFT with EGFR-TKIs. Currently ongoing clinical studies are confirming the promising therapeutic activity of second-generation EGFR TKIs and multi-targeted TKIs in several tumor types<sup>26,27</sup>. The combination of TFT with erlotinib was strongly synergistic in Caco-2 cells with a functional (e.g. w.t.) 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

## Chapter 6

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 downstream EGFR pro-survival signaling pathway and the induction of DNA damage.

Synergistic actions with EGFR tyrosine kinase inhibitors have previously been reported with cytotoxic agents such as 5-FU and TS inhibitors (pemetrexed), irinotecan and with radiation<sup>15,28,29,30</sup>. 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 tyrosine kinase inhibitors, explaining the synergistic activities. 5-FU or pemetrexed induced activation of EGFR-phosphorylation was prevented by gefitinib and erlotinib<sup>15,31</sup>. 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 TFT. TFT 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 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 TFT 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 was significantly inhibited in our cell lines. In addition, EGFR status is known not to be a predictive factor for responses to cetuximab, since responses were reported in patients with EGFR-negative tumors<sup>32,33</sup>. In addition, erlotinib has shown to have some off-target effects in leukemia cells, in which the JAK2/STAT5 pathway was inhibited<sup>34</sup>, and which were likely to be comparable to the off-target effects of gefitinib<sup>35</sup>. In the latter studies, differentiation and cell-cycle blockade induced by EGFR antagonists exerting off-target effects on AML cells were not automatically linked to an apoptotic response<sup>34,35</sup>. Our data indicate that also in colon cancer cells, erlotinib has off-target effects. 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 clinical setting.



Recent studies reported that ERGF-TKIs can decrease the expression levels and activity of TS in NSCLC, breast and colorectal cancer cells<sup>15,23,36</sup> and could be related to synergism. Since erlotinib did not inhibit TS itself in cell-free extracts<sup>15</sup>, the decrease in TS can be explained by the G<sub>1</sub>/G<sub>0</sub> cell cycle arrest that was induced by the EGFR-TKI. TS is an enzyme that is only active in the S-phase of cell cycle. TS activity was decreased in the combinations, compared to TFT alone, which is possibly related to an increased inhibition of TS and the induced cell cycle arrest<sup>15</sup>.

EGFR inhibitors have been shown to stimulate the induction of apoptosis in various cell types<sup>15,31</sup>. However, in our colon cancer cell line panel, erlotinib itself did not induce cell death as demonstrated by the sub-G<sub>1</sub> 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<sub>0</sub>/G<sub>1</sub> phase, which can be mediated by inhibition of the pro-survival signaling. Since 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<sup>15,37</sup>. Cellular damage induced by chemotherapeutic compounds can convert EGFR ligands from growth factors into survival factors for cancer cells that express functional EGFR<sup>38</sup>. 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<sup>39</sup>. Since TFT is a known inducer of DNA damage<sup>5,6</sup>, the combined treatment with erlotinib may cause persistent DNA damage induction, e.g. TFT induced DNA damage can not be repaired due to inhibition of the DNA repair mechanisms by erlotinib<sup>39</sup>. 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, since it also affects basal repair levels. Thus, the synergism found between TFT and erlotinib may be explained by a diminished ability to repair TFT-induced-DNA-damage lesions.

TP plays a role in apoptosis, cell growth and angiogenesis<sup>40</sup>. Moreover, TP may be induced after exposure to EGFR-TKIs and cytotoxic agents, which has been related to chemoresistance<sup>23</sup>. TAS-102 consists of TFT combined with TPI, a thymidine phosphorylase (TP) inhibitor<sup>3</sup>. This may be an additional advantage of a clinical combination of TAS-102 with erlotinib. Further investigation is required to elucidate the role of TP in enhancement of responses to EGFR-TKI, especially *in vivo*. In conclusion, the combination of TFT 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

## **Chapter 6**

is needed to fully elucidate this promising combination in colorectal cancer therapy, the concept of combined molecular targeting of EGFR and TFT seems to present a potential strategy in the field of molecular therapeutics.

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