Cell Cycle Modulation Enhances the Cytotoxicity of Thymidylate Synthase Inhibitors

Jennifer Sigmond, Bistra Todorova, Kees Smid, Godefridus J Peters

Department of Medical Oncology, VU University Medical Center PO Box 7057, 1007 MB Amsterdam, the Netherlands

Abstract

Thymidylate synthase (TS) is a cell cycle regulated enzyme. It increases during proliferation and has a higher activity during the S-phase in order to provide the cell with sufficient dTTP to facilitate DNA synthesis. Hence inhibition of cyclin dependent kinases (CdK) may lead to a decrease of TS and enhance the inhibition of TS. A number of CdKs control progress of the cell cycle together with checkpoint kinases (ChK1 and ChK2) which are activated by phosphorylation mediated by protein kinases such as protein kinase C (PKC). Both staurosporine (STS) and UCN-01 are inhibitors of PKC, but STS also inhibits CdK2, while UCN-01 inhibits CdK2, 4 and 6 as well as ChK1, cyclin D and pRb. We investigated the interaction between 5-fluorouracil (5FU) and STS or UCN-01 in syngeneic colon cancer cells, either wild type for p53 (LovoB2) or mutated (Lovo175x2). Cell growth inhibition was evaluated using the sulforhodamine B (SRB) test, synergism was evaluated using the multiple drug effect analysis yielding combination indices (CI: synergism < 0.9; antagonism: > 1.1), cell cycle distribution and cell death by FACS analysis, cell cycle proteins by western blots, while TS expression was measured by radioactive assays. 5FU was combined with STS or UCN-01 using simultaneous and sequential schedules. Cytotoxicity of 5FU was enhanced by UCN-01 (LovoB2, CI=0.4; Lovo175x2, CI=0.2) but less for STS (CI=0.8-0.9). At IC80 values, 5FU (5 µM) induced an S-phase arrest (2-3 fold) in both cell lines, 0.5 µM UCN-01 a slight decrease in G2-M arrest but 0.05 µM STS not. 5FU and UCN-01 combinations all decreased G2-M phase. STS and 5FU combinations led to a similar S-phase accumulation as with 5FU. Induction of cell kill after 48 hr by UCN-01 was independent of p53, as it was 2-3 fold higher (25%) in Lovo175x2 cells compared to LovoB2, for STS this was similar for both cell lines (5-10%), as well as for 5FU (2-5%). Combinations of 5FU and STS or UCN-01 resulted in additive cell kill in both cell lines. At a molecular level 5FU caused an increase in TS levels (predominantly as the ternary complex), STS downregulated TS partially, but UCN-01 completely, which was associated with a similar decrease in E2F. STS, UCN-01 and 5FU treatment also decreased TS catalytic activity in both cell lines. 5FU caused a transient appearance of pChK expression at 24 hr which was enhanced by UCN-01 and STS. In conclusion: the effect of 5FU can be enhanced by the cell cycle modulators UCN-01 and STS, by abrogation of either the S or G2M checkpoints.
Introduction

Thymidylate synthase (TS) is a rate-limiting enzyme in pyrimidine de novo deoxynucleotide biosynthesis. TS catalyzes the methylation of 2’-deoxyuridine-5’-monophosphate (dUMP) to 2’-deoxythymidine-5’-monophosphate (dTMP), with 5,10-methylene tetrahydrofolate (5-10-CH₂-THF) as a limiting cofactor (1). dTMP can be converted into 2’-deoxythymidine-5’-triphosphate (dTTP), an essential precursor for DNA synthesis. Expression of TS under physiological conditions is related to the cell cycle and has a high activity during the S-phase, but decreases when the cells do not proliferate (1). Various transcription factors and cell cycle-dependent kinases (CDK) control the increase in TS levels in the cell cycle (2). Cell cycle progress through the G1/S checkpoint is tightly regulated by cyclin/CDK complexes, which are activated by phosphorylation. Phosphorylation of various cyclins-CDKs can subsequently hyperphosphorylate the Rb and E2F complex which results in the release of E2F from phosphorylated Rb. The free transcription factor E2F can subsequently activate the transcription of several DNA synthesis-dependent proteins such as TS. TS levels are also controlled at the level of translation (3). The translation of TS-mRNA appears to be controlled by its end product, the TS protein, in an autoregulatory manner. TS protein cannot only regulate its own translation but also that of other proteins, such as p53 (4). In addition, wild-type p53 protein can also inhibit TS promotor activity (5).

5-Fluorouracil (5-FU) is an important antineoplastic agent that has proven to be effective in the treatment of colorectal cancer, stomach cancer and breast cancer. (6) The active metabolite of 5-FU, 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP) inhibits TS, which is increased in tumors compared to normal cells. TS inhibition by FdUMP is mediated by the formation of a covalent ternary complex between FdUMP, TS and 5-10-CH₂-THF (1). This leads directly to depletion of dTMP and indirectly to an accumulation of dUMP. As a result the 5-FU metabolite 5-fluoro-2’-deoxyuridine-5’-triphosphate (FdUTP) is incorporated into DNA due to lack of natural substrate dTTP. In addition, 5-fluoro-uridine-5’-triphosphate (5-FUTP), another metabolite of 5-FU can be incorporated into RNA. (1)

One of the main obstacles for the clinical use of 5-FU is the acquisition of resistance to the drug by cancer cells. In vitro studies have demonstrated that a major mechanisms of resistance is due to an increased activity of TS. This activity can have several reasons, such as gene amplification and altered autoregulation (1, 3, 7, 8). Autoregulation

Fig. 1: Proposed mechanism of action combining checkpoint abrogators with DNA damaging agents such as the pyrimidine analogue 5-FU (modified from 10);
A: DNA incorporation of drug metabolites or inhibition of TS induces an S phase arrest. Activation of Chk-1 deactivates the cdk1-cylin B complex inhibiting G2/M transition.
B: When DNA damage is combined with checkpoint abrogators such as UCN-01, cells are also arrested in the G2-M phase since UCN-01 is able to inhibit Chk1. 5-FU damaged cells go into mitosis, but the accumulated damage leads to mitotic cell death. UCN-01 and staurosporin also inhibit Cdk2/cyclinE. This leads to a lower extent of Rb hyperphosphorylation, stabilizing the complex Rb-E2F, less free E2F and less free TS, which can be inhibited more efficiently by 5-FU.
of TS-mRNA translation controls the normal level of TS protein, but binding by FdUMP disrupts this process leading to increased TS protein. TS protein can also increase due to aberrant regulation by cell cycle genes, such as Rb and E2F (9). The TS gene has an E2F binding site in its promoting region (2), leading to more synthesis of TS protein (Fig. 1). These results suggested that E2F-1 plays an important role in the acquisition of 5-FU-resistance by cancer cells (9) and that cancer therapy targeting transcription factor E2F might be effective (2).

Cell cycle perturbations are commonly observed in human malignancies. Protein kinases participate in growth factors and oncogene product-dependent signal transduction pathways and have emerged as key regulators of cell proliferation (10, 11). Staurosporine (STS) and staurosporine analogs such as 7-hydroxystaurosporine (UCN-01) are protein kinase C (PKC) inhibitors but can also inhibit other cell cycle regulating enzymes. UCN-01 has been tested both as an enhancer of cytotoxicity of other drugs and as a single cytotoxic agent (10). The potentiation of cytotoxicity by UCN-01 is assumed to be due to its ability to abrogate the S- or the G2/M-phase checkpoints (11, 12). The by-pass of these cellular checkpoints in the presence of DNA damage would lead the cell to death. At the molecular level, it was found that UCN-01 also inhibits chk1 kinase, which leads to activation of cdc25C phosphatase and inhibition of Wee1 kinase, resulting in increased Cdc2/cyclin B1 activity (11). UCN-01 induces apoptosis in various human tumor cell lines. The precise mechanism of UCN-01 antitumor activity is still not completely understood (10, 11, 12, 13). UCN-01 does not interact with DNA in both cell-free and cellular systems nor causes single-strand or double strand breaks in vitro (10). It was however clearly shown, that the induction of apoptosis by UCN-01 does not correlate with its ability to inhibit PKC activity (10). UCN-01 can inhibit activation and phosphorylation of protein kinase B (Akt), in vitro and in vivo (10). Inhibition of Akt activity correlated with UCN-01 induced apoptosis, thus suggesting that UCN-01 may exhibit its cytotoxicity in part by turning off the Akt survival pathway. In vitro and in vivo, UCN-01 selectively blocks cell cycle progression from G1- to S-phase and preferentially induces G1-phase accumulation of the target cells. UCN-01 induced G1-phase accumulation is associated with inhibition of cdk2 kinase. Inhibition of cdk2 kinase takes place by the induction of cdk inhibitors such as p21^{cip1} and p27^{kip1} or, as it was hypothesized, by interaction of UCN-01 with the ATP binding site of cdk2 (12) (Fig. 1). It was postulated that G1-phase arrest resulting from inhibition of cdk2 activity might be associated with apoptosis induced by UCN-01.

In this study we investigated the potential of UCN-01 and staurosporine to increase the cytotoxicity of the DNA damaging agent 5-FU. For this purpose we used the colon cancer cells LovoB2 (wild type p53) and Lovo175x2 (mutant p53). Mechanistic studies focused on interaction between TS and cell cycle proteins, leading to cell cycle disturbance and cell death.

Materials and Methods

Materials

5-FU, UCN-01 and staurosporine were purchased from Sigma Chemical Co (St Louis, MO, UK). [5-^3H]dUMP (TRK-287; specific activity 19 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). The primary antibody mouse antihuman TS (clone TS 106) was from Labvision, Neomarker, the primary monoclonal E2F-1, recognizing the first 368 amino acids was from SantaCruz, the polyclonal rabbit antihuman phospho-Chk1 (Ser 345) antibody was from Cell Signaling Technology (USA), while the secondary antibodies-goat anti-rabbit IgG HRP was from Santa Cruz Biotechnology (USA) and the polyclonal goat anti-mouse-from Dako A/S (Denmark). Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

Cell culture

The source, characteristics and culture conditions of the colon cancer cell lines, LovoB2 (wild type p53) and Lovo175x2 (mutant p53) were described earlier (5). Briefly cells were cultured at 37°C in 5%CO2 in DMEM medium supplemented with 10% fetal calf serum and 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES).

Anti-proliferative effects of 5-FU, staurosporine, UCN-01 and their combinations on LovoB2 and Lovo 175x2 colon cancer cells were evaluated using the sulforhodamine B
(SRB) assay, as described earlier (14). Briefly cells were plated in 96-wells plates; after 24 hr one plate (d 0) was processed as control, to the other plates drugs were added and processed after 72 hr. For the combinations a fixed ratio was used of 5-FU and the PKC inhibitors (100:1) with concentrations ranges of 0.1-50 µM for 5-FU and 0.001-0.5 µM for staurosporine/UCN-01. For sequential combinations, UCN-01 or staurosporin were first added, followed after 24 hr by 5FU. In order to evaluate the combinations the multiple drug effect analysis was used as described earlier (15); the interaction between drugs was expressed as the Combination Index (CI). Values obtained at fraction affected (FA) of 0.5, 0.75 and 0.9 were averaged and used to calculate the means of at least 3 separate experiments. A mean CI < 0.9 was considered as synergistic, > 1.1 as antagonistic and 0.9 – 1.1 as additive (15).

**Cell cycle changes and induction of apoptosis**

After 24 hr and 48 hr drug exposure of cells using IC80 concentrations (final concentrations: 5 µM 5-FU, 0.5 µM UCN-01, 0.05 µM staurosporine), adherent and floating cells were harvested and used for cell cycle analysis and cell death (sub-G1) using Fluorescence-activated cells sorter (FACS) flow cytometer (Becton Dickinson) based on double stranded DNA binding properties of propidium iodide as described earlier (16). The programme Cellquest was used for analysis.

**Western Blot analysis**

In order to evaluate expression of TS and the cell cycle proteins E2F and Phospho-Chk1, cells were harvested and prepared for western blot analysis as described earlier (16). The target was visualized by means of ECL(+) detection as described.

<table>
<thead>
<tr>
<th>IC50 (µM)</th>
<th>Lovo B2 (wild-type)</th>
<th>Lovo 175x2 (mut-p53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporine</td>
<td>0.003 + 0.0006</td>
<td>0.005 + 0.0009</td>
</tr>
<tr>
<td>UCN-01</td>
<td>0.02 + 0.002</td>
<td>0.091 + 0.014</td>
</tr>
<tr>
<td>5-FU</td>
<td>1.86 + 0.38</td>
<td>3.98 + 0.79</td>
</tr>
</tbody>
</table>

Sensitivity to the drugs was determined using the SRB assay after 72 hr to the drugs. Values are means + SE of at least 3 separate experiments

**TS-catalytic assay**

In order to evaluate the effect of the drugs, cells were exposed to 5 µM 5FU, 0.5 µM UCN-01 or 0.05 µM staurosporin alone or in combination. Cells were harvested after 24, 48 and 72 hr, and the TS catalytic activity was determined at 1 µM dUMP as described earlier (17).

**Results**

**Drug sensitivity and combinations**

![Fig. 2: Example of the synergistic interaction between 5FU and UCN-01 in Lovo175x2 cells. Cells were exposed simultaneously to 5FU and UCN-01 for 72 hr, after which the SRB assay was performed to evaluate growth inhibition. Synergism was determined using the multiple effect analysis. The broken indicates the cut-off between antagonism (CI>1.1) and synergism (CI<0.9), showing a clear synergism at FA>0.3 and very synergistic at FA~0.7.](image)

Table 1: Sensitivity of Lovo variants to 5FU, staurosporine and UCN-01.

<table>
<thead>
<tr>
<th>Simultaneous</th>
<th>Sequential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lovo</td>
<td>Lovo 175x2</td>
</tr>
<tr>
<td>UCN-01 + 5FU</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Staurosporin + 5FU</td>
<td>0.79 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means + SD of at least 3 separate experiments. Cells were exposed to the drugs simultaneously for 72 hr, or sequentially (24 hr UCN-01 or staurosporin, followed by the combination of UCN-01 + 5FU or staurosporin + 5FU for 48 hr. |
The sensitivity to 5-FU, staurosporine and UCN-01 was determined after 72 hr exposure to the drugs (Table 1). The wild-type cells were more sensitive to 5-FU and staurosporin, but not to UCN-01. The effect of the combinations was studied using simultaneous and sequential combinations. All combinations were synergistic when cells were exposed to the drugs simultaneously; the staurosporin combination was moderately synergistic (CI values about 0.8-0.9), but that with UCN-01 was highly synergistic with CI values varying from 0.2-0.4 (Table 2, Fig 2). Sequential combinations were less synergistic, being additive or even antagonistic in the mutp53 Lovo175x2 cells.

**Cell cycle changes and induction of apoptosis**

Since staurosporin and UCN-01 are cell cycle directed drugs, we determined their effect, alone and in combinations, on cell cycle distribution (Fig. 3). In both untreated Lovo variants 45-60% of the cells were in G1 phase, 10-15%-in S-phase and 30-40% -in G2/M phase. UCN-01 caused an accumulation of cells in the G2/M phase (60% after 24 hr, decreasing to 40% after 48 hr). Staurosporin, however, increased accumulation of cells to 50-60% in the G1 phase, which was more pronounced in the Lovo wild type cells. 5FU induced a clear accumulation of cells in the S-phase (30% after 24 hr and 40% after 48 hr). In the simultaneous combinations the 5FU induced an accumulation of cells in the S-phase (30% after 24 hr and 40% after 48 hr). In the sequential combinations the 5FU induced a S-phase accumulation after 24 hr seemed to be predominant, which was even more pronounced in the mutp53 cells, both for the staurosporin and UCN-01 combinations. However, after 48 hr this effect was less. For the sequential combinations the effect of the first drug seemed to be most important for the cell cycle distribution, i.e.
with 5FU first, the cell cycle distribution after 48 hr resembled that of 5FU alone (S phase accumulation), but with staurosporin first more cells accumulated in the G0-G1 phase, while with UCN-01 first more cells accumulated in the G2-M phase.

Both staurosporin and UCN-01 already induced a clear cell kill after 24 hr, increasing after 48 hr (Fig. 4). Especially for UCN-01 cell kill was more pronounced in the mut p53 cells. Cell kill induced by 5FU was moderate, even after 48 hr. In the combinations with staurosporin the simultaneous combination was less effective in wt p53 cells. In the sequential combinations, 5FU first was most effective. For UCN-01 combinations a higher cell kill (up to 35%) was observed in the mut p53 cells compared to less then 15% in the wt cells. The extent of cell kill was independent of the sequence.

**Expression of TS and upstream proteins-E2F-1 and pChK1**

In order to get more insight in the mechanism of the drug interactions we determined expression of TS and the cell cycle proteins E2F and pChK1. Treatment of LovoB2 cells for 24 hr with 5FU reduced the expression of free TS by trapping TS in the ternary complex detected at 38 kD (Fig. 5A). The total amount of TS increased as well. Staurosporin alone did not affect TS expression at 24 hr, but UCN-01 alone decreased the expression of TS at 24 hr. At 48 hr TS was undetectable after treatment with UCN-01 or staurosporin. Both staurosporin and UCN-01 decreased TS expression. In the combination initial trapping of TS in the ternary complex was not affected by staurosporin or UCN-01 at 24 or 48 hr (Fig. 5A).

In order to explain the downregulation of TS, we also determined the expression of E2F and pChK1, which play a crucial role on G1-S and G2-M transition, respectively. Both staurosporin and UCN-01 downregulated the expression of E2F after 24 hr (Fig. 5B), but the effect of UCN-01 was more pronounced. Also in the combination with 5FU, E2F was downregulated, indicating the formation of the ternary complex was independent of E2F. When 5FU preceded staurosporin or UCN-01 the downregulation of E2F after 48 hr was less then when 5FU was given after staurosporin or UCN-01.
The expression of pChk1 expression was undetectable in control samples of LovoB2 and Lovo175x2 cells. Staurosporin and UCN-01 did not increase the expression of pChk1, but 24 hr 5FU treatment did, both alone and in combination with staurosporin or UCN-01. However, after 48 hr the expression was undetectable again.

**Effect on TS levels**

Since TS inhibition is crucial in the efficacy of 5FU we determined the catalytic TS activity in LovoB2 and Lovo175x2 cells (Fig. 6). TS activity in LovoB2 cells was clearly decreased in staurosporin and UCN-01 treated cells, while at the used 5FU concentration TS inhibition was moderate, as found earlier (17).
concentration was used, because at a moderate inhibition, a modulating effect can be studied. In the combination of 5FU with staurosporin an intermediate effect was observed, which was less in staurosporin treated cells. In Lovo175x2 cells the results were comparable.

**Discussion**

This study shows that modulation of 5FU by cell cycle abrogation can enhance the cytotoxicity of the combination, not only in wt p53, but the effects of UCN-01 were even more pronounced in mut p53 cells. These effects were clearly related to cell cycle abrogation of the compounds.

Both 5FU and the PKC inhibitors staurosporin and UCN-01 can exert cytotoxic effects. 5FU is a potent inhibitor of TS by formation of a ternary complex (1). The efficacy of 5FU can be enhanced by an increase of TS inhibition, which can be achieved by either decreasing the levels of TS or stabilizing the ternary complex. TS is a cell cycle dependent enzyme, and regulated by the transcription factor E2F (2). Hence deregulation of the cell cycle by a decrease in E2F can decrease TS expression. Indeed in wtp53 cells UCN-01 decreased E2F as well as TS expression, preventing cells to accumulate in the S-phase; however, in mut p53 cells, 5FU induced accumulation of cells in the S-phase is partly prevented when UCN-01 preceded 5FU. Under these conditions more cell kill is observed, possibly because cells can not continue to cycle since UCN-01 also inhibits the G2-M checkpoint. Phosphorylation of Chk1 was observed in cells exposed to 5-FU possibly as a response to the DNA damage caused by 5-FU. Since UCN-01 inhibits Chk1 kinase (11), this leads to activation of cdc25 phosphatase preventing phosphorylation of cdk1/cyclin B1. 5FU damaged cells go into mitosis and die. In the case of staurosporin, mitosis is not inhibited since cells accumulate in the G0-G1. 5-FU damaged cells go into the S-phase, but will be prevented to cycle further after staurosporin treatment.

Further evidence for a dual cytotoxic effect, mediated by 5FU induced DNA damage and cell cycle abrogation, is shown by the effect on TS activity and expression. A decrease in TS levels was seen both by western blotting and activity assays. For 5FU this was a clear inhibition as seen by the ternary complex formation, but for UCN-01 this was clearly a decrease in E2F leading to a decrease in TS. For staurosporin this effect was delayed and only seen after 48 hr. Hence we saw a dual effect on TS, one which can be explained partly by the autoregulation of TS mRNA translation as proposed by Liu et al (3), or stabilization of TS as proposed by Kitchens et al (18). The effect of UCN-01 in relation to TS is an agreement with other studies showing that transfection of E2F decoy ODN (oligodeoxynucleotides) resulted in decreased TS mRNA expression (9).

In conclusion, cytotoxicity of 5FU can be enhanced by the PKC inhibitors staurosporin and UCN-01. However, cell kill by the combination with staurosporin is also mediated by prevention of cell cycling, leading to enhanced DNA damage. In contrast, cell kill mediated by UCN-01 be related to a mitotic arrest.

**References**


