Effect of staurosporine and ucn-01 on gemcitabine cytotoxicity in relation to cell cycle effects and p53 status

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Abstract
Gemcitabine (dFdC) is an anti-cancer agent that is affected by cell cycle modulation. Staurosporine and 7-hydroxyxtaurosporine (UCN-01) are potent protein kinase C (PKC) inhibitors as well as inhibitors of cyclin dependent kinase 2 (cdk2) and were therefore investigated for potential synergism, cell cycle modulation, cell death induction, and the role of the p53 protein. Lovo colon cancer cell line variants with wild type and mutant p53 (Lovo 175x2) or inactive (Lovo Li) were used for this purpose.

Combinations of dFdC with staurosporine were most synergistic when cells were exposed to dFdC prior to staurosporine, while for UCN-01 the simultaneous exposure was most synergistic. This synergism appeared to be related with abrogation of the cell cycle and cell cycle proteins. For dFdC (1.0 μM), a gradual time dependent increase of cells in S phase (up to 40%) was observed in all Lovo variants. Staurosporine (0.05 μM) initially induced accumulation of cells in the G2/M phase (after 24 hr), while 48 hr exposure showed accumulation in the S-phase. UCN-01 (0.5 μM) caused an arrest in G0/G1 after 24 hr exposure, while after 48 hr cells accumulated in the S phase. Simultaneous exposure to dFdC combinations showed an average cell cycle distribution of both drugs when used alone. In sequential addition of dFdC combinations, the first drug dominated the cell cycle distribution.

Synergism of gemcitabine combinations was associated with induction of cell death by UCN-01, which was 2-fold higher in Lovo 175x2 (mutant p53) compared to other Lovo variants. Additive effects in induction of cell death were observed at the simultaneous addition of dFdC and UCN-01, but exposure to dFdC prior to UCN-01 caused a 2-fold higher induction of cell death than the sum of each compound alone in Lovo 175x2 cells, in contrast to the other lines. Accumulation of cells in the G2/M phase prevented repair of DNA damage, resulting in increased apoptosis. These data demonstrate that staurosporine and UCN-01 affect dFdC cytotoxicity via modulation of the cell cycle.

Keywords: gemcitabine, staurosporin, UCN-01, protein kinase C, p53, E2F, cell cycle, cell death

Introduction
Gemcitabine (dFdC) is widely used for treatment of solid tumors and almost always combined, either with cytotoxic drugs such as cisplatin, paclitaxel and 5-fluorouracil (5-FU) or targeted drugs such as erlotinib [1]. dFdC is a deoxynucleoside analogue, in which the 2’ hydrogens of the nucleoside deoxycytidine are replaced by fluoride. dFdC needs to be phosphorylated by deoxycytidine kinase (dCK) to become active. The main mechanism of action is incorporation of its active metabolite dFdC triphosphate (dFdCTP) into the DNA [2]. Furthermore, dFdC diphosphate (dFdCDP) inhibits ribonucleotide reductase (RNR), an essential enzyme that is involved in DNA synthesis [3]. RNR is responsible for the reduction of ribonucleotides to their corresponding deoxynucleotides that will be further phosphorylated to deoxynucleoside triphosphates (dNTPs). Inhibition of RNR is one of the self-potentiating properties of dFdC since this will lead to depletion of dNTP pools, increasing the dFdC incorporation into the DNA [4]. Combination therapies are rationally designed in order to increase the efficacy of the separate drugs and circumvent development of resistance. E.g. the combination of cisplatin and gemcitabine is based on a decreased repair of DNA-platinum adducts as well as an increased dFdC incorporation into DNA. Following preclinical data on the synergism of dFdC with cisplatin [5,6], this combination is now considered to be one of the most active schedules against NSCLC and bladder cancer [1,7].

Hence, increasing the efficacy of a combination will benefit most from the use of agents that work at different targets, inducing additive effects or ideally synergistic interactions. DNA damaging agents such as dFdC that
arrest cells in the S phase are suitable agents to be combined with cell cycle inhibitors that act on different cell cycle phases thereby increasing the therapeutic response. The protein kinase C (PKC) inhibitors staurosporine and its analogue UCN-01 are agents that induce cell cycle arrest in preclinical experimentst. Staurosporine is an indolo (2,3-a) carbazole derived from bacterium Streptomyces which turned out to be a potent but non-selective inhibitor of PKC. Staurosporine induces G1 [8] and G2 arrest [9] depending on the concentration [10,11]. Staurosporine is a potent inducer of cell death. [9,12]. Since staurosporine inhibits signal transduction pathways that regulate growth factor response, proliferation and apoptosis, and some preclinical results showed promising antitumor effects, new analogues were developed with higher selectivity for PKC, such as 7-hydroxy-staurosporine (UCN-01). UCN-01 exhibited a potent anti-tumor activity in both in vitro and in vivo tumor models [13]. Besides PKC inhibition, staurosporine and UCN-01 can affect kinases that are related to PKCs such as cyclin dependent kinases (cdks), which are involved in cell cycle progression. E.g. Kawakami demonstrated an inhibitory effect on cdks 2, 4 and 6 in NSCLC cell line A549 [14]. This decline increased levels of cdk inhibitors p27 and p21 members of the Cip/Kip family. Together with a decrease in cycline D3 levels with concomitant reduced phosphorylation of the retinoblastoma (Rb) gene all contribute to the induced G1 phase arrest by UCN-01 [15,16,17]. UCN-01 also induces cell death in cell lines that lack p53 function while the induced cytotoxicity is p53 independent [18,19].

In this study, we evaluated the combination of dFdC with staurosporine and UCN-01 focusing on growth inhibition, cell death induction, cell cycle and the role of the p53 protein. Lovo colon cancer cell line variants with wild type (Lovo B2), non-functional (Lovo Li) and mutant (Lovo 175x2) p53 were used for this purpose.

Materials and methods

Drugs and biochemicals

Dulbecco’s Modified Eagle’s Medium (DMEM) was obtained from Flow Laboratories (Irvine, Scotland). Both fetal calf serum (FCS) and Hank’s balanced salt solution (HBSS) were purchased from Gibco Europe (Paisly, UK). Gemcitabine (Gemzar, dFdC) was provided by Eli Lilly Research Laboratories, (Indianapolis, IN, USA). UCN-01 was kindly provided by Dr. Bob Schultz at the NCI (Bethesda, MD USA). Staurosporine and propidium iodide were from Sigma Chemical Co (St Louis, MO, UK). Hybond Enhanced Chemoluminescence (ECL) nitrocellulose membranes, Hyperfilm ECL plus and ECL detection kit were obtained from Amersham International (Buckinghamshire, UK). Mouse monoclonal antibodies against human P53 (Clone Ab-2) and against E2F1 (clone KH-95) were from EMD Biosciences, Darmstadt, Germany, and from Santa Cruz Biotechnologies, respectively. The secondary antibody goat-anti-mouse was from DAKO (Glostrup, Denmark). Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

Cell lines

Lovo is a solid human colon cancer cell line. Lovo colon cancer cell line variants with wild type (Lovo B2), non-functional (Lovo Li) and mutant (Lovo 175x2) p53 with a mutation at position 175 (Arg-His) leading to gain of function [20] were grown as monolayers and were cultured in DMEM medium, containing 10% FCS and 20 mM HEPES at 37°C under an atmosphere of 5% CO2.

Growth inhibition studies

Growth inhibitory effects of dFdC, Staurosporine and UCN-01 were evaluated with the sulforhodamine-B (SRB assay) as described earlier [21]. Shortly, cells of Lovo variants (5000 cells/well) were exposed to various drug concentrations for 72 hrs. Thereafter, cellular protein was precipitated, fixed and stained with SRB and the optical density was measured at 540 nm. The interactions were studied at a fixed ratio based on the IC 50 concentrations of the drugs using the multiple drug effect analysis software program developed by Chou and Talalay [22], (Calcusyn software, BioSoft, Ferguson, MO, 1996) which enables to determine drug interactions, given by the Combination Index (CI), in which a CI >1.1 represents antagonism, 0.9<CI<1.1 additivity and CI<0.9 synergism. CI values are plotted against fraction affected (FA) resulting in CI-FA plots; a FA of 0 is normal 100% growth, FA=0.5 represents the IC50 value and a FA of 1 represents 100% growth inhibition. In the CI-FA plot the CI values at FA >0.5 are evaluated and per experiment only the CI values at FA 0.5, 0.75 and 0.9 were averaged. Different treatment schedules were used; simultaneous exposure and sequential exposure with dFdC as the first drug and PKC inhibitors as the second drug added 24 hr after the first drug without refreshing the medium.

FACS analysis

Cell cycle distribution and induction of apoptosis was analysed by FACSScan (Becton Dickinson, Mount View, CA) using Propidium Iodide (PI) as DNA binding agent. After drug treatment, adherent and floating cells were harvested and counted. After centrifugation (1200 rpm, 5 min), the pellet was gently resuspended in 1 ml hypotonic propidium iodide (PI)-solution (50 μg/ml PI, 0.1% sodium citrate, Triton X-100, 0.1 mg/ml ribonuclease A) to a concentration of 5×10 5–10 6 cells/ml in round-bottomed fluorescent activated cell sorting (FACS) tubes. Cells were analysed by using Cellquest software (Becton Dickinson, Mount View, CA).

Western blot analysis

Frozen cell pellets were lysed in buffer containing 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris HCl pH 7.6, and 5 mM EDTA. Protein content was measured using the
BioRad assay. For determination of protein expression, 25 µg protein was loaded and separated on a 10% SDS-PAGE gel (acrylamide:bis 29:1), followed by blotting on a nitrocellulose membrane. Blocking occurred overnight at 4°C with 5% milk powder (Protifar) in TBS-T (TBS (20 mM Tris pH 7.6 and 150 mM NaCl) mM + 0.05% Tween-20). Membranes were incubated for one hr with the primary antibodies (dilution 1:100). Subsequently the membranes were incubated with HRP conjugated anti mouse or anti-rabbit antibodies (dilution 1:1000). After each incubation step the nitrocellulose membranes were washed several times with TBS-T. The HRP was visualized by means of ECL(+) detection solution and autoradiography and quantified by scanning on a GS-690 Bio-rad scanner (Bio-Rad, Hercules, California, USA). Levels of expression were reported relative to the untreated control samples [23].

Results

Growth inhibition studies

In order to study the role of p53 mutations on sensitivity to and modulation by staurosporine and UCN-01 we used three Lovo colon cancer variants (Lovo B2, empty vector plasmid control; wild-type p53), Lovo Li (functionally inactive p53) and Lovo 175x2 (transfected with mutated p53). Lovo 175x2 was less sensitive to all three drugs although this difference was minor for staurosporine (Table 1).

The combination indices of dFdC with staurosporine or UCN-01 are summarized in Table 2. The simultaneous combination of dFdC with UCN-01 was synergistic in all Lovo variants. Furthermore, the highest synergistic effect of the combination with UCN-01 was found in mut p53 Lovo 175x2 compared to the other Lovo variants (Figure 1). Also for the combination of dFdC with staurosporine strong synergism was observed in Lovo 175x2 but not in Lovo Li cells, while in Lovo B2, the combination was not additive. Because exposure to dFdC prior to staurosporin or UCN-01 induced more cell death than vice versa, we also studied this sequence, by adding staurosporin and UCN-01 24 hr after dFdC. The combination with UCN-01 was also synergistic in Lovo Li and Lovo 175x2 at sequential exposure although to a lesser extent than the simultaneous combination. However, in Lovo B2 cells the sequential combination with UCN-01 even changed to additive and that with staurosporin to synergism, while the effects in Lovo Li and Lovo175x2 hardly changed.

Table 1. Sensitivity of Lovo variants to gemcitabine, staurosporine and UCN-01. Values are means ± SE of at least 3 separate experiments.

|        | Lovo B2 | Lovo Li | Lovo 175x2 |
|--------|---------|---------|------------|
| IC₅₀ (nM) Gemcitabine | 4 ± 1 | 0.8 ± 0.2 | 22 ± 4.8 |
| IC₅₀ (nM) Staurosporine | 3 ± 0.6 | 2 ± 0.6 | 5 ± 0.9 |
| IC₅₀ (nM) UCN-01 | 20 ± 2 | 15 ± 2.3 | 91 ± 14 |

Sensitivity to the drugs was determined using the SRB assay with 72-hr exposure.

Cell cycle analysis

Representative data on cell cycle distribution of combinations of dFdC and staurosporine or UCN-01 are shown in Figure 2. No clear differences were observed between the Lovo variants regarding the effect of the drugs on cell cycle distribution. For dFdC (1.0 μM), a gradual increase (about 2-fold) of cells in S phase was observed in all Lovo variants. Exposure to staurosporine (0.5 μM) initially induced accumulation (from 35 to about 60%) of cells in the G₂/M phase (after 24 hr), which shifted after 48 hr exposure to the S-phase. An arrest in G₀/G₁ was observed after 24 hr exposure UCN-01 (0.5 μM), while

Table 2. Combination indices for combinations of simultaneous (sim) and sequential (seq) exposure of gemcitabine (dFdC) followed by UCN-01 or staurosporine (STS) in Lovo variants

| Combination | Lovo B2 | Lovo Li | Lovo 175x2 |
|-------------|---------|---------|------------|
| dFdC / UCN-01 (1:1) sim | 0.39 ± 0.08 | 0.47 ± 0.11 | 0.17 ± 0.04 |
| dFdC -> UCN-01 seq | 0.91 ± 0.28 | 0.57 ± 0.05 | 0.45 ± 0.15 |
| dFdC/STS (1:1) sim | 1.17 ± 0.34 | 1.06 ± 0.20 | 0.44 ± 0.02 |
| dFdC -> STS seq | 0.32 ± 0.10 | 0.87 ± 0.28 | 0.53 ± 0.26 |

Evaluation of possible synergism using the median drug effect analysis method of Chou and Talalay (Biosoft). CI is given as the mean of CI values which represent the synergistic effect calculated for growth inhibition of 50, 75 and 90%. Symbols: ++++ strong synergism, +++ synergism, + slight synergism, + nearly additive, - slight antagonism. Results are means ± SEM of at least 3 separate experiments.
after 48 hr cells accumulated in the S phase (up to almost 2-fold). Simultaneous exposure to dFdC combinations showed an average cell cycle distribution of each drug alone. In sequentially given drug combinations, the cell cycle distribution of the first drug added was dominant.

**Induction of cell death**

Induction of cell death was determined by analysis of the sub-G1 fraction of the FACS data. Cell death induction was investigated after treatment for 72 hr with dFdC combinations (Table 3). In Lovo B2 and Lovo Li cells dFdC induced more cell death than in Lovo 175x2. However, in Lovo 175x2 cells with mutant p53 UCN-01 and staurosporine induced more cell death compared to the other Lovo variants. The combination of dFdC with UCN-01 produced more cell death in Lovo 175x2 compared to Lovo Li and Lovo B2.

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**Figure 1.** Representative growth inhibition curves of the simultaneous combination of gemcitabine (GEM) with UCN-01 in a fixed molar ratio in LovoB2 cells.

**Figure 2.** Cell cycle distribution of Lovo B2, Lovo Li and Lovo 175x2 after treatment with combinations of 1.0 μM gemcitabine (dFdC) with 0.5 μM UCN-01 or 0.05 μM STS. Cells were exposed to drugs simultaneously for 24 or 48h (+ bars with one time point) or sequentially (STS > dFdC or dFdC > STS; means 48h exposure to the first drug while the last 24 hr the second drug was also present. Values are means of 3 separate experiments.
This is related with the increased induction of cell death by UCN-01 in Lovo 175x2 compared with the other Lovo variants. A more than additive cell kill of 48 hr exposure by dFdC followed by staurosporine or UCN-01 was observed in all cell lines. However, the most striking synergistic effect was observed in Lovo 175x2. Induction of cell death was 2-fold higher compared to the additional effect of used drugs alone. Furthermore, a more than additive cell kill was also observed after treatment of Lovo 175x2 cells with simultaneous addition of dFdC and UCN-01.

### p53 and E2F protein expression

Since for UCN-01 it has been reported that it may bypass P53 dependent cell death we investigated its expression in the three lines upon drug exposure. As expected in Lovo Li P53 was not induced, while in Lovo 175x2 the high expression was not affected (data not shown). However, in Lovo B2 single drug exposure to dFdC upregulated p53 about 4-fold at 48 hr, but after exposure to STS or UCN-01 a slight decrease was found (Figure 3). In simultaneous drug exposure, drugs contributed equally to p53 expression leading to about 2-fold upregulation. In sequential drug exposure, the first drug given dominated in the p53 induction, leading to no change in p53 expression when UCN-01 or STS were added first, although a high extent of cell death was found in this sequence. Since also in the Lovo 175x2 cells a similar and higher extent of cell death was found than in the wt p53, induction of cell death follows a p53 independent pathway.

E2F expression showed a differential expression in the combinations. In Lovo 175x2 it was only induced in the sequential combinations of UCN-01 (Figure 4). For dFdC we found a small decrease and for STS a small increase. In the STS combinations E2F was decreased when dFdC preceded STS. In the Lovo B2 cells UCN-01 and STS increased E2F expression. But in the sequential combinations it was decreased when dFdC preceded STS or UCN-01.

### Discussion

Combinations of dFdC with UCN-01 or staurosporine are most effective when cells were exposed to dFdC prior to staurosporine or UCN-01. Apparently the cell cycle...
effects as induced by the first drug, prevented cells to cycle further and cells were forced into apoptosis which was most pronounced for the dFdC and UCN-01 combination in the cells with mutant p53, in contrast to the other lines. Staurosporine and UCN-01 have been developed as protein kinase Cα inhibitors, but have multiple other cell cycle targeted effects. We demonstrated earlier in non-small cell lung cancer cells that potentiation by staurosporine is independent of PKC inhibition, but associated with a decrease of the cell cycle regulator E2F and an even more pronounced decrease of the gemcitabine target ribonucleotide reductase [23]. UCN-01 is even less specific for PKC and is a known abrogator of cell cycle proteins, such as cdk 2 and 4, the G2/M and S checkpoint via “checkpoint kinase (chk)” chk1 and possibly chk2. [24]. Hence low concentrations of UCN-01 cause S phase arrest progressing into the G2 phase before going into apoptosis, while high concentrations cause immediate S->M transition [25].

Abrogation of the G2/M checkpoint is an attractive target when it is combined with DNA damaging drugs. DNA damage triggers the protein kinases ataxia telangiectasia (ATM) and ATM-Rad3-related (ATR) to activate chk1 and chk2, inactivating the phosphatase cdc25c by phosphorylation, which will prevent cyclineB-cdk1 to be dephosphorylated, arresting cells in G2-M transition of cells to repair the induced DNA damage. In cells with wild-type p53, DNA damage will stimulate p53, leading to an S-phase arrest and allowing cells to repair DNA damage. In mut-p53 cells this regulation is abrogated, so that UCN-01 by inhibition of the G2/M checkpoint regulators, will increase DNA damage. Moreover UCN-01 will further prevent DNA repair and enhance the effect of deoxynucleoside analogues ara-C, dFdC, S-FU [26-31], the purine analogue fludarabine [26,32] and DNA binding agents such as cisplatin, thiotepa, mitomycin C, cisplatin, melphalan and topotecan [26]. Treatment with a DNA damaging drug (cisplatin, 5FU, nucleoside analogs or radiation) prior to UCN-01 or a novel Chk1 inhibitor (SCH00766) was synergistic and induced more cell death than vice versa through abrogation of the G2/M checkpoint and was associated by reduced expression of cyclins A and B and activation of Cdk1 [33,34].

UCN-01 and dFdC combinations induced more cell death in p53 mutant Lovo 175x2 cells than wt p53 cells. In addition to dFdC this holds for NSCLC cells treated with cisplatin or the topoisomerase I inhibitor SN38. In wt p53 Lovo B2 the induction was additive since UCN-01 led to a G1 arrest and in the combination to a decrease in E2F and not to the abrogation of the G2/M checkpoint. The

Figure 4. Expression E2F in Lovo B2 and Lovo 175x2 cells, after 48 h exposure of 1.0 μM gemcitabine (gem), 0.5 μM UCN-01 or 0.05 μM staurosporine (STS). Simultaneous exposure (drug + drug) and sequential exposure of drugs were used (drug > drug; the second drug was added 24 hr after the first drug). The 24h control (Con) was set on 100%, E2F expression was corrected with that of b-actin.
observation that in cancer cells with inactive p53, post treatment with a checkpoint inhibitor such as UCN-01 enhanced sensitivity to DNA damaging agents is an excellent approach for combination therapy. Indeed most cancer cells have mutations in the G1/S checkpoint (such as the tumor suppressor genes p53 and Rb) and only exceptionally cancer cells have defects in the G2/M checkpoint.

Kroep et al showed that the activity of dCK is correlated with dFdC sensitivity of xenograft mouse models [35]. In NSCLC cells treatment with staurosporin increased dCK activity, possibly by a mixture of a direct effect [23] or via post-translational regulation, such as protein phosphorylation. Indeed, activation of dCK may involve a phosphorylation-dependent step, since okadaic acid and calyculin A, broad-spectrum inhibitors of serine/threonine-specific protein phosphatases, significantly increased dCK activities [36,37]. In contrast, λ protein phosphatase digestion abolished the dCK activity of cell extracts [38]. In addition, Smal et al., demonstrated that recombinant; His-tagged dCK can be phosphorylated [39]. The synergistic effect of dFdC with staurosporin or UCN-01 in colon cancer cells may partly be similar to that in NSCLC and due to downregulation of free E2F, an upregulation of dCK and a downregulation of ribonucleotide reductase. Since UCN-01 clearly interferes with cdks, the action of both UCN-01 and staurosporine in combination with dFdC seems also to be related to cell cycle effects by preventing cells to progress in cell cycle after dFdC exposure leading to more cell death.

In conclusion, combinations of dFdC with UCN-01 or staurosporine were most effective when cells were exposed to dFdC prior to staurosporine or UCN-01. Targeting different cell cycle phases and abrogation of cell cycle checkpoints are both attractive strategies in combination with DNA damaging agents since the sequential combination of DNA damaging agents prior to checkpoint abrogators such as UCN-01 and novel analogs, prevent repair of DNA damage leading to cell death.

Competing interests
The authors declare that they have no competing interests.

Authors’ contribution
The main part of the paper has been designed and written by J Sigmond and GJ Peters. J Kamphuis and L Leon contributed to part of the paper and wrote some parts, while A Bergman took part in the supervision and writing as well.

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