Cell cycle effects of fatty acid derivatives of cytarabine, CP-4055, and of gemcitabine, CP-4126, as basis for the interaction with oxaliplatin and docetaxel

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Abstract. To bypass resistance due to limited entry into the cell derivatives of cytarabine (CP-4055, elacytarabine) and gemcitabine (CP-4126) containing a fatty acid chain at the 5' position of the nucleoside were developed. CP-4055 showed an increased retention of the active metabolite, the triphosphate. This characteristic was supposed to favor combinations, such as with the tubulin antagonist docetaxel, the platinum oxaliplatin and the antifolate pemetrexed. The role of the cell cycle effects of CP-4055 and CP-4126 on the efficacy of the combination with docetaxel or pemetrexed was determined. The combination of CP-4055 with oxaliplatin and docetaxel was also evaluated in a mouse xenograft model. CP-4055 induced a G2/M and S phase accumulation and CP-4126 an S phase accumulation. Both analogs induced a dose-dependent cell kill (apoptosis and necrosis). None of the docetaxel combinations induced a synergistic effect. The combination of docetaxel with CP-4055 or CP-4126 induced a G2/M accumulation in the A549 (lung cancer) cell line, but a G0/G1 accumulation in the WiDR (colon cancer) cell line. Preincubation with docetaxel induced an increased cell kill in both cell lines. The combination with oxaliplatin showed a synergistic effect in both cell lines. Combinations with pemetrexed were antagonistic in both cell lines. In the A549 cell line pemetrexed with CP-4055 led to an increase of the G0/G1 phase and the S phase. In WiDR the combination of pemetrexed with CP-4055 increased the G0/G1 phase and increased the cell kill. Pemetrexed with CP-4126 induced an increase in the G0/G1 phase and the S phase in the A549 cell line. In the xenograft study, on a colon cancer and a lung metastasis model, the combination of CP-4055 with docetaxel showed the best results. Treatment with CP-4055 followed by docetaxel after 4 h resulted in a reduction in metastasis in a lung metastasis model, and a favorable toxicity profile was observed. In conclusion, the combinations with oxaliplatin showed a synergistic effect in the combination studies. Although the combinations with docetaxel did not show an enhanced effect in the *in vitro* studies, this combination revealed an increased effect in the xenograft model.

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Introduction

The deoxynucleoside analogs cytarabine (Ara-C) and gemcitabine (dFdC) are drugs commonly used in the treatment of a variety of cancers, although cytarabine is restricted for use in hematological malignancies (1). The rate-limiting step in the activation of both drugs to their active triphosphate forms is the conversion to the intermediate monophosphate by deoxycytidine kinase (dCK) (1,2). Both drugs can be inactivated by deoxycytidine deaminase (dCDA). The second intermediate of dFdC, dFdC diphosphate, is known to inhibit ribonucleotide reductase, which is essential in providing deoxyribonucleotides required for DNA repair (3). DNA polymerisation is stopped after incorporation of dFdC triphosphate into DNA and one additional nucleotide, this masked chain termination prevents removal of gemcitabine by exonucleases (4). Ara-C triphosphate incorporation into DNA also causes chain termination, while the active metabolite of Ara-C can inhibit DNA polymerase by competitive inhibition (5). Due to their hydrophilic nature these drugs are dependent on the equilibrative and concentrative nucleoside transporters (hENT and hCNT) to cross the cell membrane into the cell (6,7). In order to enhance the uptake of the drugs into the cell by making the drugs less hydrophilic, derivatives containing a fatty acid side chain have been developed. The fatty acid was esterified to the 5' position on the sugar moiety. The derivatives of gemcitabine (CP-4126) and cytarabine (CP-4055, elacytarabine) each contain a fatty acid with a chain length of eighteen carbon atoms and one trans-double bond (elaidic acid) in position 9. CP-4055 has shown remarkable antitumor activity in various solid cancer xenografts in which the parent drug Ara-C has no activity (8). Other studies

(9-11) have demonstrated that CP-4055 is still dependent on activation by dCK, but that the drug has a longer cellular retention and a different effect on DNA and RNA synthesis compared to Ara-C. CP-4126 was also able to inhibit the deamination of dFdC. CP-4126 is currently in phase I clinical studies in solid tumor patients (intravenous and oral administration). CP-4055 has shown acceptable tolerability in phase I studies (12) and is currently being tested in phase II studies on solid tumors and leukemia. CP-4055 has shown additive to synergistic antiproliferative activity in combinations with gemcitabine, irinotecan, topotecan, cloretazine and idarubicine (13). Ara-C and dFdC are usually applied in combinations, but the efficacy of these combinations is sometimes limited by their hydrophilic nature or limited accumulation of the triphosphate. Therefore we investigated whether the lipophilic derivatives would be equally or even more effective in combinations with oxaliplatin and docetaxel.

Oxaliplatin is a platinum analog that, like cisplatin, forms intra- and interstrand DNA, DNA-protein and protein adducts. Adducts formed by oxaliplatin in the DNA form primary lesions that block DNA replication and transcription (14). Oxaliplatin is used in the treatment of colorectal cancer (15). Gemcitabine combined with cisplatin is one of the most active combinations in NSCLC (16) for which very pronounced synergism was found for *in vitro* and *in vivo* combinations with gemcitabine-cisplatin (17,18) and gemcitabine-oxaliplatin (19-22).

Docetaxel promotes the polymerization of tubulin. The microtubules formed in the presence of docetaxel are very stable; this stability causes disruption of microtubule dynamics. As microtubule dynamics is essential for cell division and interphase processes, disruption of microtubule dynamics leads to cell death (23).

Pemetrexed is a multitargeted antifolate that works by inhibiting several key enzymes involved in cellular metabolism (24). Docetaxel and pemetrexed are used in the treatment of lung cancer (25,26). Additive to synergistic effects were found for combinations of gemcitabine with docetaxel (27) and pemetrexed (28).

Since gemcitabine and pemetrexed are standard therapy for NSCLC and oxaliplatin for colon cancer, we investigated whether the lipophilic derivatives would enhance the antiproliferative activity of these drugs.

Materials and methods

Drugs. Pemetrexed (PMX) was obtained from the pharmacy department of the VU University Medical Center. Docetaxel (Doc) and oxaliplatin (OHP) were obtained from Sanofi-Aventis (Paris, France). CP-4055 and CP-4126 were obtained from Clavis Pharma (Oslo, Norway).

Cell lines. To test the activity of the drugs *in vitro*, we selected a non-small cell lung cancer cell line (A549) (29), a colon cancer cell line (WiDR) (30) and a variant of the WiDR cell line that has been cultured under low folate conditions (WiDR-LF). Normal cell culture medium contains supraphysiological folate levels, which reduce the effect of antifolates. So WiDR-LF was used because the folate levels in low folate conditions are more representative for folate levels in human plasma and therefore these data could be more predictive for the activity of antifolates than results obtained from cell lines cultured in high folate medium (30). The parent cell lines were cultured in DMEM medium with glutamine (BioWhittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (Gibco, New York, NY, USA) and 20 mM HEPES buffer (BioWhittaker). The WiDR-LF cell line was cultured with dialyzed serum and 2.5 nM leucovorin (Sigma-Aldrich, St. Louis, MO, USA).

Chemosensitivity assay. As chemosensitivity assays we used the sulforhodamine-B (SRB) assay and the (4,5-dimethyl-thiazol-2,5-diphenyl) tetrazolium bromide (MTT) assay (31). Since antifolates tend to increase the size of cells, thus increasing the protein content, the MTT assay was used in the experiments with PMX. For the other experiments the SRB assay was used. Cells were transferred to 96-well plates; on day one a serial dilution of one drug was added to the cells. Different combinations were used in this study, such as a fixed ratio of both drugs and a serial combination of one drug combined with a constant concentration of the second drug. When using a 4-h preincubation the drug in a constant concentration was always added 4 h before adding the combination of a serial dilution and a constant concentration, so the serial dilution is incubated for 72 h and the constant concentration was incubated for a total of 76 h. When using a combination based on the IC_{50} ratio of the drugs, both drugs were added in a serial dilution. The relative amount of cells at drug addition was determined by fixating control wells at day 0 (drug addition) and processed as described below. The other (treated and control) cells were fixed after the incubation period, washed and stained with SRB. The stained proteins were measured at 492 nm with an automated spectrophotometric microplate reader (Tecan, Salzburg, Austria); the measured optical density correlates with the amount of cells at the moment of fixation. When using the MTT assay the cells were incubated for 3 h with MTT, DMSO was added to dissolve the crystals and the plates were measured at 492 nm.

The data were plotted in a graph to give a growth inhibition curve. From this growth inhibition curve the IC_{50} value was determined by interpolating at the 50% growth level.

Median-drug effect analysis. The median-drug effect analysis method (32) has been widely used for evaluation of potential synergism between different drugs (33,34). The Calcusyn software (Calcusyn 1.1.1, Biosoft, Cambridge, UK) developed by Chou and Talalay was used for this evaluation. Doses are calculated by the formula: D=Dm[Fa/(1-Fa)]1/m; where Dm is the dose required for 50% growth inhibition, Fa is the fraction affected and m is the slope. The combination index (CI) was calculated using: CI=[(D)1/(Dx)1]+[(D)2/(Dx)2]+[a(D)1(D)2/(Dx)1(Dx)2]; where a=1 for mutually non-exclusive drugs, (D)1 and (D)2 are the doses of the separate drugs and the combination, and (Dx)1 and (Dx)2 are the doses resulting in a growth inhibition of x%.

A CI of <0.9 indicates synergism, of 0.9-1.1 additivity and of >1.1 antagonism. Experimental conditions that result in a Fa of <0.5 are of dubious importance. The aim is to obtain a significant effect, which means at least >50%, but preferably complete, growth inhibition. Hence, in *in vitro* models, only the interval between Fa 0.5 and 0.95 is likely to be of interest (35). Per experiment a mean CI of the data points with a Fa higher than 0.5 was calculated and used for overall evaluation.

Cell cycle distribution and cell kill. In 6-well plates 5x10^4 cells were seeded and grown for 24 h. After 24 h one drug was added at the IC_{50} concentration and combined with the IC_{25} concentration of the other drug, similar to the cytotoxicity assay. After 72 h incubation the cells were harvested and analyzed by incubation with a propidium iodide solution (PI); containing 50 μg/ml PI, 0.1% (Tri-) sodium citrate, 0.1% Triton X-100 and 0.1 mg/ml RNase A (Qiagen, Hilden, Germany). The PI-stained cells were measured on a flow cytometer (FACScan, BD Benelux, Erembodegem-Aalst, Belgium) and the results were analyzed with the Cellquest software. The accumulation in the G1, S and G2/M phase was described as fraction of the total accumulation in these phases of the cell cycle. Accumulation in the SubG1 and tetraploidy/debris was described as fraction of the total distribution. The sub G1 fraction was evaluated as the dead cell population (both apoptotic and necrotic).

Platinum-DNA accumulation. The experimental procedure was based on a method described by van Moorsel *et al* (36). Briefly, cells were treated for 24 h with 200 μM OHP alone or combined with the IC_{25} concentration of CP-4055 or CP-4126, after incubation the cells were washed and harvested. DNA was isolated using the QIAamp DNA minikit (Qiagen) and treated with RNAse (Qiagen) to remove all residual RNA. DNA content and quality was estimated with a nanodrop (Nanodrop technologies, Wilmington, DE, USA). A total of 0.1 volume sodium chloride (1.65 M) was added to the dissolved DNA. A calibration curve was made using different concentrations of OHP (0-1.5 μM) in TE-buffer containing 0.165 M sodium chloride. Platinum content of samples and standards was determined using Zeeman atomic absorption spectrometry (AAS) (Varian SpectrAA-10 atomic absorption spectrometer; Varian, Palo Alto, CA, USA).

Xenograft study. Combinations of CP-4055 together with OHP and Doc were tested in mouse models (37). Housing and all procedures involving animals were performed according to protocols approved by the animal care and use committee, in compliance with the German Animal Protection Law. 5776 human colon carcinoma was used because earlier it was characterized for its sensitivity to gemcitabine, CP-4126 and CP-4055 (unpublished data). Tumor fragments were transplanted to Ncr:nu/nu mice. The murine Lewis Lung (LL) carcinoma is an established tumor in Crl:BDF1 female mice; this model was used because it will cause lung metastases, and has been used earlier to investigate synergism of gemcitabine with cisplatin and docetaxel (38,39). Endpoints for all but the LL were toxic death, body weight, tumor volume, white blood cell count (WBC) and thrombocyte count. For the LL cell line the endpoints were toxic death, body weight, lung weight, lung metastasis, WBC and red blood cell count. Each experiment consisted of 6-8 groups with 8 mice per group. One group was treated with the vehicle (PBS); the others with: 1) CP-4055 at maximum tolerated dose (MTD), 2) CP-4055 at MTD combined with drug 2 at MTD, 3) CP-4055 at MTD combined with drug 2 at 50% MTD, 4) CP-4055 at MTD combined with drug 2 at 25% MTD, 5) CP-4055 at 50% MTD combined with drug 2 at 50% MTD and 6) CP-4055 at 25% MTD combined with drug 2 at 25% MTD.

Table I. Sensitivity of the cell lines to the single drugs used in the combination experiments.

Drug	WiDR	WiDR-LF	A549
CP-4055	0.76±0.21	0.76±0.21	0.26±0.05
CP-4126	0.01±0.003	0.01±0.003	0.008±0.001
OHP	2.36±0.43		2.29±0.51
Doc	0.0014±0.0003		0.001±0.0002
PMX	0.06±0.006	0.02±0.005	0.23±0.06

Values are IC_{50} (μM) means ± SEM of three separate experiments. IC_{50} values were determined after 72-h exposure to the drugs.

Results

The IC_{50} values for the single drugs are given in Table I. These data were used as a basis for the combination experiments.

In order to determine at which concentrations CP-4055 and CP-4126 induced significant cell cycle effects, we determined the concentration dependency for these drugs. CP-4055 induced a G2/M and S phase accumulation in the A549 cell line; S phase accumulation at the lower concentrations and G2/M accumulation at the higher concentrations in the WiDR cell line (Fig. 1). At the highest concentration a different effect was observed, this was possibly because a lot of cells did not survive the 72-h exposure to these high concentrations of drugs. In both cell lines CP-4055 induced dose-dependent cell kill (Fig. 2b) and in the A549 cell line it also induced tetraploidy (Fig. 2c). CP-4126 increased S phase accumulation and dose-dependent cell kill in both cell lines (Figs 1 and 2a).

Because a sequential effect of gemcitabine combined with taxanes was observed previously (38,40); Doc was combined with CP-4055 or CP-4126 in different schedules. A preincubation of 4 h with CP-4055 or CP-4126 did not increase the sensitivity to docetaxel (Table II), similar to previous data found with paclitaxel with gemcitabine (38). No increased effect was observed after preincubation with Doc prior to CP-4055. In the combination with CP-4126 preincubation with docetaxel increased the sensitivity from antagonistic to additive.

Combinations with OHP both induced a synergistic effect in both cell lines (Fig. 3). The combination with CP-4055 gave CI values of 0.8 and 0.7 in the WiDR and A549 cell line respectively, while the combination with CP-4126 gave values 0.9 and 0.7.

All combinations with PMX were antagonistic in both cell lines with all different combinations that were tested (Table II). PMX in a serial dilution and CP-4055 or CP-4126 in a fixed concentration induced antagonism in both cell lines.

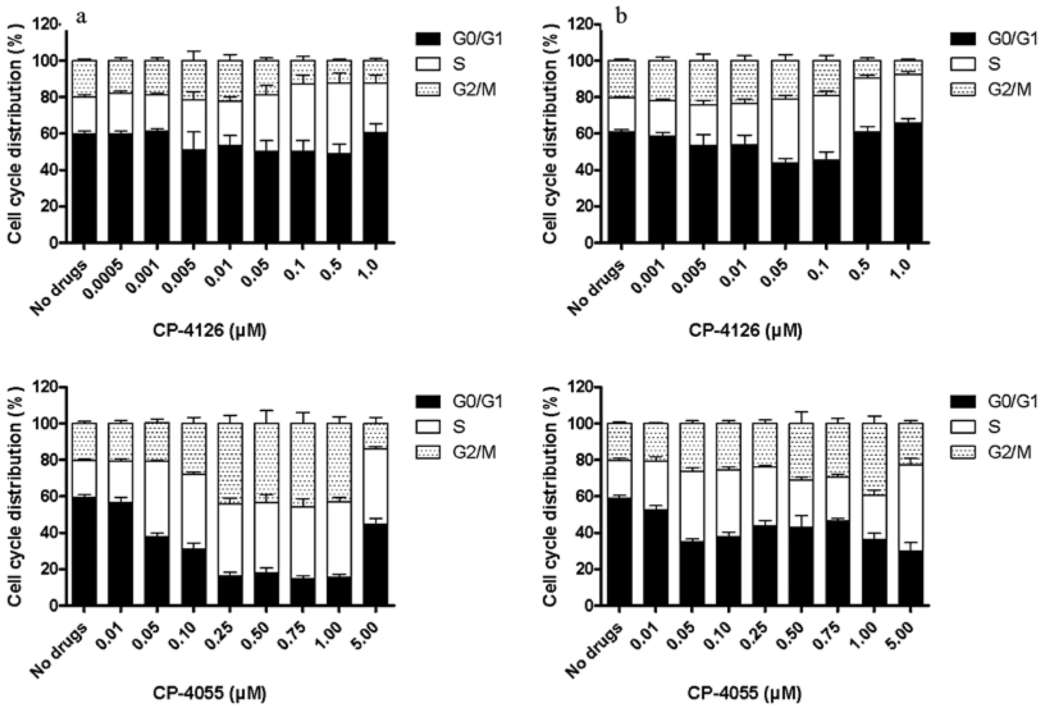

Figure 1. Dose-dependency of CP-4126 or CP-4055 on the cell cycle in the (a) A549 and (b) WiDR cell lines. Cells were exposed for 72-h incubation to increasing concentrations of drugs. Values represent percentiles of at least three separate experiments; SEM was <9.8% of the mean.

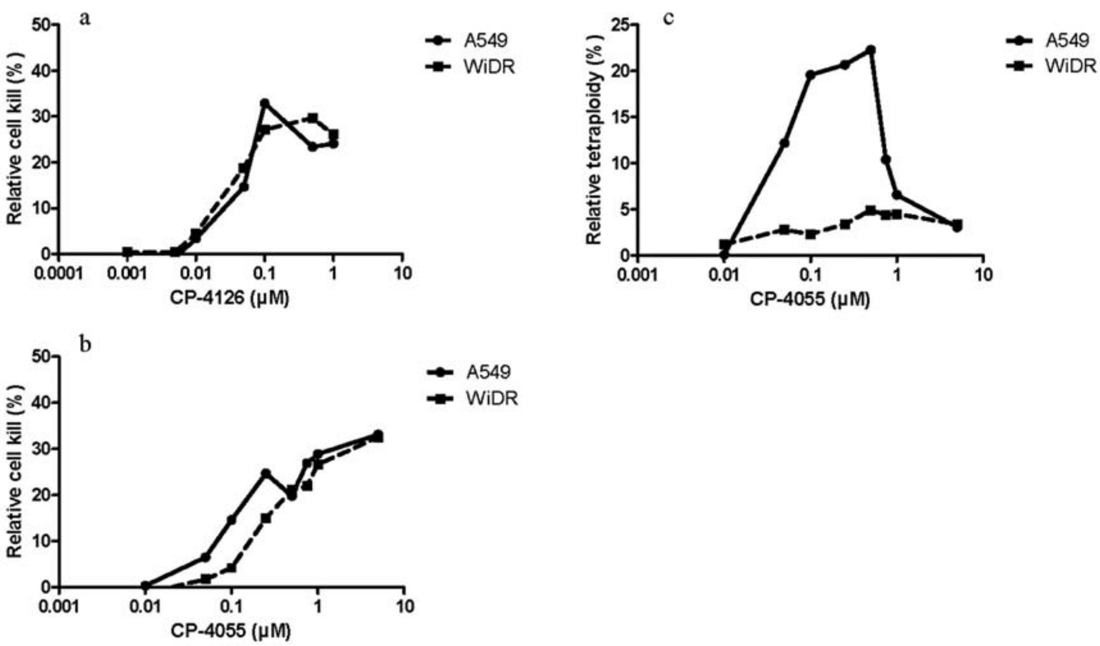

Figure 2. Dose-dependency of CP-4126 or CP-4055 on cell kill (Sub G1, which includes apoptosis and necrosis) of (a) CP-4126 or (b) CP-4055, and the effect of (c) CP-4055 on the tetraploidy (no effect of CP-4126 on tetraploidy was observed). Cells were exposed for 72 h-incubation to increasing concentrations of drugs. Values represent percentiles of at least three separate experiments; SEM was <6.9% of the mean.

In the low folate cell line a slight improvement was observed for the combination with CP-4126, while the opposite was observed with the combination with CP-4055. Adding both PMX and CP-4055 or CP-4126 in a fixed ratio did also not increase the effect.

Since scheduling affected the interaction of CP-4055 and CP-4126 with PMX and Doc, we investigated whether their cell cycle effects would give insight into the mechanism. The combinations with PMX did not show an increase in cell kill or tetraploidy and the cell cycle data demonstrated that the combination had an effect very similar to PMX alone (Fig. 4). In the A549 cell line Doc combined with CP-4055 increased the accumulation of cells in the G2/M phase by 7% (Fig. 4). However, with a preincubation of CP-4055 the increase was

Table II. Mean combination indices of the different combination experiments with OHP, PMX and Doc.

Combination	Serial dilution	Fixed (IC_{25})	WiDR	A549	WiDR-LF
PMX+CP-4126	PMX, CP-4126[a]	-	3.5±0.7	2.1±0.8	
PMX+CP-4055	PMX, CP-4055[a]	-	1.5±0.3	1.6±0.4	
PMX+CP-4126	PMX	CP-4126	1.6±0.4	1.7±0.6	1.2±0.6
PMX+CP-4055	PMX	CP-4055	1.1±0.1	2.1±0.2	3.1±0.3
Doc+CP-4126	Doc	CP-4126	1.6±0.1	1.3±0.4	
Doc+CP-4055	Doc	CP-4055	1.3±0.2	1.1±0.3	
Doc+CP-4126	Doc	CP-4126[b]	1.7±0.5	2.3±1.1	
Doc+CP-4055	Doc	CP-4055[b]	2.2±0.3	1.5±0.4	
CP-4126+Doc	CP-4126	Doc[b]	1.0±0.2		
CP-4055+Doc	CP-4055	Doc[b]	1.5±0.04		
OHP+CP-4126	OHP	CP-4126	0.9±0.2	0.7±0.2	
OHP+CP-4055	OHP	CP-4055	0.8±0.2	0.7±0.3	

[a]Both drugs were added in a serial dilution, in a fixed 1:1 ratio (IC_{50}:IC_{50}). [b]4-h preincubation before adding the combination. Values represent mean CI values ± SEM of at least three separate experiments.

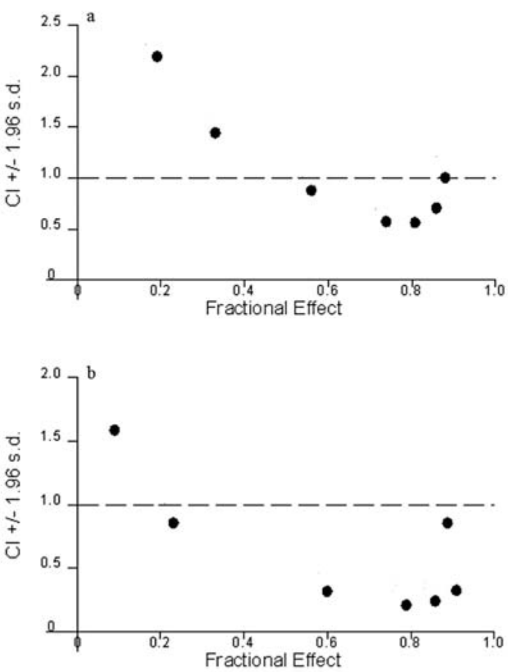

Figure 3. Representative fractional effect-combination index (CI) graph of the combination of (a) OHP combined with CP-4055 and (b) OHP combined with CP-4126 in the A549 cell line. OHP was added in a serial dilution and CP-4055 and CP-4126 at a fixed IC_{25} concentration; (a) 0.0035 and (b) 0.004 μM. A CI of <0.9 indicates synergism, of 0.9-1.1 additivity and of >1.1 antagonism. The mean CIs of three separate experiments in A549 were: 0.7±0.2 for CP-4126 and 0.7±0.3 for CP-4055, and in the WiDR cell line: 0.9±0.2 and 0.8±0.2, respectively.

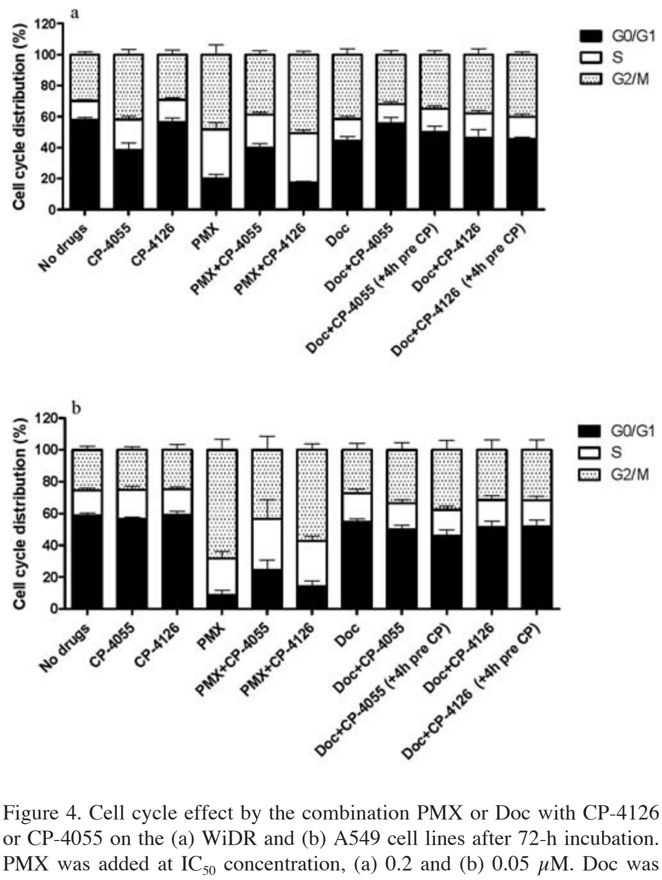

Figure 4. Cell cycle effect by the combination PMX or Doc with CP-4126 or CP-4055 on the (a) WiDR and (b) A549 cell lines after 72-h incubation. PMX was added at IC_{50} concentration, (a) 0.2 and (b) 0.05 μM. Doc was added at IC_{50} concentration, (a) 0.01 and (b) 0.008 μM, CP-4126 at IC_{25} concentration (0.004 μM) and CP-4055 at IC_{25} concentration, (a) 0.0035 and (b) 0.15 μM. Values represent percentiles ± SEM of at least three separate experiments.

found to be 11%. In the WiDR cell line Doc together with CP-4055 increased the G0/G1 phase accumulation by 11% but by preincubation with Doc this was reduced to 5%. In the A549 cell line Doc together with CP-4126 increased the accumulation of cells in the G2/M phase by 4%. Surprisingly, in both cell lines both combinations increased the sub G1 phase, which was higher after preincubation compared to simultaneous exposure (Fig. 5). In the A549 cell line preincubation with CP-4055 increased cell kill from 7%

Table III. Combinations of CP-4055 with OHP in the 5776 colon carcinoma.[a]

Compound	Treatment (days)	Dose (mg/kg)	Toxic deaths (days)	BWC[b] (%)	Optimum T/C[c] (%)	WBC[d] (10^6/ml)	Thrombo (10^6/ml)
PBS	13-17		0/8	-2		6.3±1.4	1122±117
CP-4055	13-17	30	0/7	-3	45 (27)	4.7±1.6	1161±136
	13-17	15	0/7	-5	63 (23)	4.6±2.2	1136±126
OHP	13-15	2.5	6/8 (18-22)	-19	ne	1.6±1.1[e]	812±240
	13-15	1.25	0/7	-13	11 (27)[e]	4.6±2.3	957±231
CP-4055	13-17	30	0/8	-21	10 (34)[e]	2.1±0.7[e]	843±170
OHP	13-15	1.25					

[a]Mice were injected IP with CP-4055 on days 13-17, and with OHP on days 13-15. OHP was administered 4 h after CP-4055. [b]Body weight change; [c]relative tumor volume, treated to control; [d]white blood cell count; ne, not evaluable. [e]Significant to PBS.

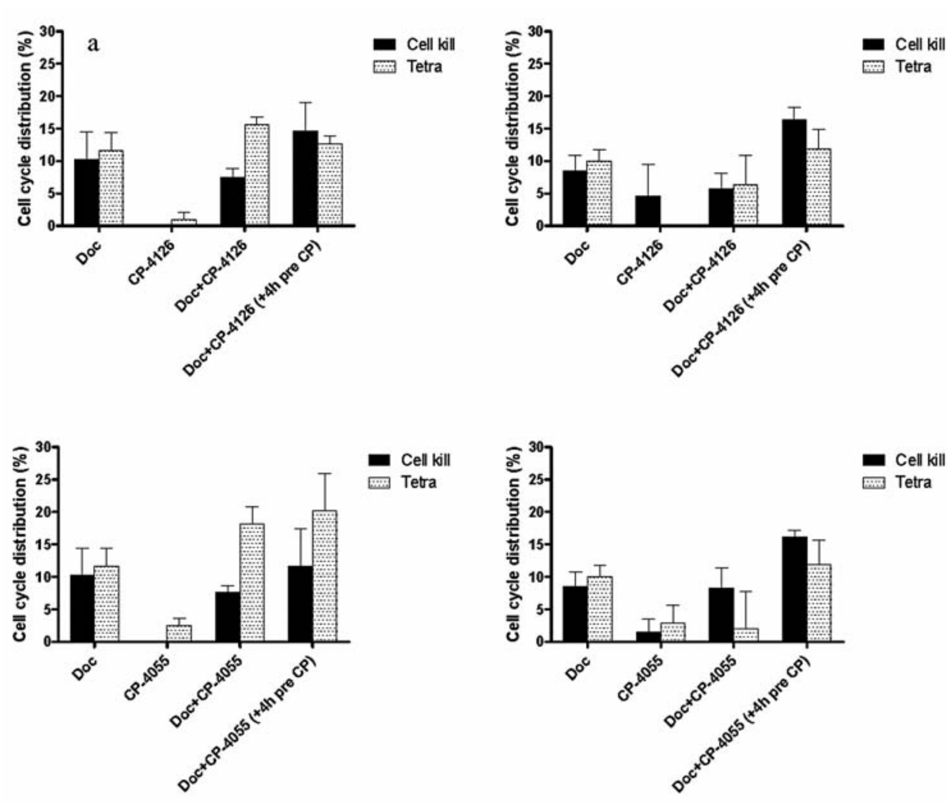

Figure 5. Effect on cell kill (Sub G1, which includes apoptosis and necrosis) and tetraploidy by the combinations of Doc with CP-4126 or CP-4055 on the (a) A549 and (b) WiDR cell lines after 72-h incubation. Doc was added at IC_{50} concentration, (a) 0.01 and (b) 0.008 μM, CP-4126 at IC_{25} concentration (0.004 μM) and CP-4055 at IC_{25} concentration, (a) 0.0035 and (b) 0.15 μM. Values represent percentiles ± SEM of at least three separate experiments. Observed effects were not significant.

(simultaneous) to 12% (preincubation), whereas in the WiDR cell line preincubation with CP-4055 doubled the amount of cell kill. This was also observed with preincubation of CP-4126 in both cell lines. In A549 and WiDR both combinations increased tetraploidy, while the increase was the highest after preincubation with CP-4055.

For mechanistic studies on OHP we focused on the DNA adduct formation. Compared to OHP alone (0.68 pmol/μg DNA) platinum DNA adduct formation was only slightly increased in the WiDR cell line with the combination of OHP together with CP-4126 (0.95 pmol/μg DNA; not significant). For the other combinations no increase in adduct formation was observed.

Several combinations were also tested in *in vivo* model systems, in which we first investigated which drug could be given at its MTD in combination. The major toxicity observed

Table IV. Combinations of CP-4055 with OHP in the LL lung carcinoma.[a]

Compound	Treatment (days)	Dose (mg/kg)	Toxic deaths (days)	BWC[b] (%)	Lung weight (g)±SD	Lung mets/mouse[c]	WBC[d] (10^6/ml)	RBC[e] (10^9/ml)
PBS			0/8	3	0.56±0.25	++++	14.7±2.8	10.3±0.6
CP-4055	3-7	30	0/8	3	0.51±0.15	++++	10.3±0.9	9.6±0.3
	3-7	15	0/8	3	0.62±0.12	++++	10.6±1.8	9.6±0.4
OHP	3-7	2.5	0/8	1	0.59±0.15	++++	4.9±0.9	9.2±0.4
	3-7	1.25	0/8	4	0.63±0.19	++++	10.9±2.5	9.6±0.6
CP-4055	3-7	30	0/8	4	0.48±0.17	++++	7.6±2.7[g]	9.0±0.4
OHP	3-7	1.25						
CP-4055	3-7	15	1/8	-5	0.34±0.1[f]	+++	2.4±0.8[g]	9.8±0.3
OHP	3-7	5	(13)					
CP-4055	3-7	15	0/8	1	0.49±0.1[f]	++++	5.2±1.6[g]	9.6±0.1
OHP	3-7	2.5						

[a]Mice were injected IP with CP-4055 on days 3-7, and also with OHP on days 3-7. OHP was administered 4 h after CP-4055. [b]Body weight change; [c]++++ >20 nodes, +++ 10-20 nodes; [d]white blood cell count; [e]red blood cell count; [f]significant to CP-4055 (unpaired, two tailed t-test); [g]significant to PBS.

Table V. Combinations of CP-4055 with Doc in the LL lung carcinoma.[a]

Compound	Treatment (days)	Dose (mg/kg)	Toxic deaths (days)	BWC[b] (%)	Lung mets/mouse[c]	WBC[d] (10^6/ml)	RBC[e] (10^9/ml)
PBS	3-7,10-14		0/8	5	+++(+)	15.1±2.4	10±0.4
CP-4055	3-7 10-14	25	0/8	4	++++	8±1.6	9.2±0.3[f]
Doc	3, 7, 11	15	0/8	5	++(+)	12.2±3.4	8.9±0.3[f]
	3, 7, 11	7.5	0/8	3	++	12.8±2.9	9.4±0.3[f]
CP-4055	3-7 10-14	25	0/8	-1	++	6.4±1.4[f]	8.8±0.4[f]
Doc	3, 7, 11	7.5					
CP-4055	3-7 10-14	12.5	0/8	3	++	9.7±3.4[f]	9.2±0.6[f]
Doc	3, 7, 11	7.5					

[a]Mice were injected IP with CP-4055 on days 3-7 and on days 10-14, and with Doc on days 3, 7 and 11. Doc was administered 4 h after CP-4055. [b]Body weight change; [c]++++ >20 nodes, +++ 10-20 nodes, ++ 6-10 nodes; [d]white blood cell count; [e]red blood cell count. Significant to *CP-4055, +Doc, [f]PBS (unpaired, two tailed t-test).

in mice treated with OHP as a single drug was leucopenia, while Doc induced erythropenia and CP-4055 induced leucopenia in the Crl:BDF1 mice. The combination of CP-4055 and OHP induced leucopenia in both the Ncr:nu/nu and the Crl:BDF1 mice, but toxicity was not acceptable when combined at the MTD of OHP. The MTD was reached in the combinations of CP-4055 (30 mg/kg) with OHP (2.5 mg/kg) and in the combination of CP-4055 (15 mg/kg) with OHP (5 mg/kg). The combination of CP-4055 with Doc induced leucopenia and erythropenia, the level of leucopenia appeared to decrease when using different treatment schedules.

The combination of CP-4055 with OHP in the colon 5776 model showed a similar effect to OHP alone at its MTD (Table III). The combination with OHP in Lewis Lung did not show a reduction in the amount of metastasis and also no improvement over single drug treatment (Table IV). In the

Table VI. Combinations of CP-4055 with Doc in the LL lung carcinoma.[a]

Compound	Treatment (days)	Dose[b] (mg/kg)	Toxic deaths (days)	BWC[c] (%)	Lung mets/mouse[d]	WBC[e] (10^6/ml)	RBC[f] (10^9/ml)
PBS	3-7, 10-14		0/8	4	++++	14.6±2.9	10.6±0.3
CP-4055	3-7 10-14	30	0/8	3	++++	14.1±2.5	9.6±0.3[g]
Docetaxel	3, 7, 11	20	0/8	5	+++	10.2±2.9	9.7±0.6[g]
CP-4055	3-7 10-14	25 (am)	0/8	-5	++[g]	9.2±2.1[g]	9.0±0.4[g]
Docetaxel	3, 7, 11	15 (am)					
CP-4055	3-7 10-14	25 (am)	0/8	-3	++[g,h]	10.2±1.1[g]	8.9±0.3[g]
Docetaxel	3, 7, 11	15 (pm)					
Docetaxel CP-4055	3, 7, 11 3-7 10-14	15 (am) 25 (pm)	0/8	3	+++[g]	10±1.7[g]	9.5±0.5[g]

[a]Mice were injected IP with CP-4055 on days 3-7 and on days 10-14, and with Doc on days 3, 7 and 11. [b]am: injection in the morning, pm: injection in the afternoon; [c]body weight change; [d]++++ >20 nodes, +++ 10-20 nodes, ++ 6-10 nodes; [e]white blood cell count; [f]red blood cell count. Significant to [g]PBS, [h]CP-4055.

Lewis Lung model Doc and CP-4055 as single drugs did not affect the number of metastasis (Table V and Fig. 6). The combination of both drugs caused a reduction in the number of lung metastases. Different treatment schedules showed that there was a reduction in the number of lung metastasis. The best result was obtained with the combination of CP-4055 first and Doc after 4 h (Table VI and Fig. 6).

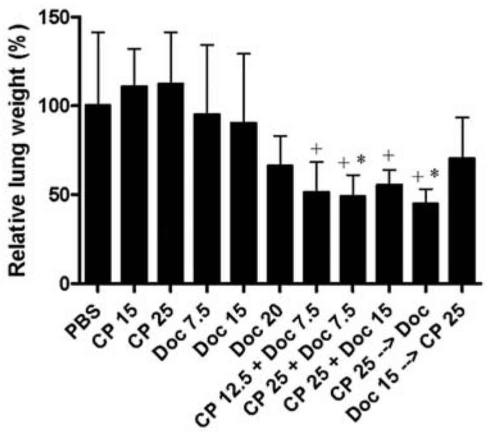

Figure 6. Lung weight in LL carcinoma lung metastasis model after treatment with the single drugs and the combinations. Values represent means ± standard deviation of 8 mice. The values of control mice are set at 100% and those of treated mice are relative to this. See Tables V and VI for dose and schedule. Significantly different compared to: [+]CP-4055, [*]docetaxel (unpaired, two tailed t-test, p<0.05).

Discussion

The lipophilic fatty acid analogs of cytarabine (CP-4055) and gemcitabine (CP-4126) were evaluated for a potential synergism with several clinically active cytotoxic drugs. A synergistic effect with OHP and an additive effect with Doc were observed *in vitro*. Evidence suggests that different cell cycle effects formed the basis for these drug interactions. At least additive effects for combinations with CP-4055 were observed *in vivo*.

Both CP-4055 and CP-4126 showed a concentration-dependent disruption of the cell cycle. CP-4055 induced G2/M and S phase accumulation, and subsequent cell kill. CP-4126 demonstrated an S phase accumulation similar to the cell cycle effect observed with gemcitabine (41); it also induced a major amount of cell kill. As deoxynucleoside analogs act by incorporation into DNA they generally affect the S phase of the cell cycle (42). The G2/M accumulation induced by CP-4055 was observed with the higher concentrations (>IC_{50}); at this high concentration part of the damaged cells might progress to the next phase without the damage being properly repaired. The downstream G2/M checkpoint will in turn cause the cells to be arrested. In the A549 cell line there was also a large amount of tetraploidy possibly associated with the highest amount of disruption of the cell cycle. It has been shown that after treatment with DNA-damaging agents the HCT116 human colon carcinoma cell line failed in chromosomal segregation after entry into mitosis (43).

The combination of OHP with CP-4126 showed a synergistic effect in both cell lines. OHP is part of the standard treatment of colon cancer and cisplatin of NSCLC; there is some evidence of clinical activity of the combination of OHP and gemcitabine in NSCLC (19-22). This indicates that the combination of CP-4126 with OHP might be eligible for further clinical development. The combination of OHP with CP-4055 also showed a synergistic effect in both cell lines. Because Ara-C is not active in solid tumors there is no evidence of clinical activity of the combination with OHP. However, since CP-4055 is active in solid tumor xenografts in which Ara-C is inactive (8), CP-4055 should be considered as a new drug in clinical development. Accumulation and retention of Ara-CTP, the active metabolite, showed a favorable profile compared to Ara-C alone (Adema, *et al*, Proc Am Assoc Cancer Res 99th Annual Metting, vol. 49: abs. 5740, 2008).

The analogs did not increase the amount of platinum DNA adduct formation; this is possibly due to very low concentrations of the analogs compared to the OHP concentration. Similarly the doses of both OHP and CP-4055 had to be decreased in the combination *in vivo* because of additive toxicity, resulting in a similar antitumor effect as OHP alone.

Combinations with Doc showed that preincubation with the derivatives did not improve the antiproliferative effect. However the cell kill data (apoptosis and necrosis) indicate that preincubation increased the amount of cell kill to levels higher than the levels caused by Doc alone. This sequential effect of gemcitabine combined with taxanes was observed previously (38,40); where taxane preincubation also increased the amount of cell kill. A sequential effect was also observed in the xenograft study, where the preincubation with CP-4055 showed the highest pharmacological activity both concerning antitumor effect and toxicity. Taking this evidence together it seems that to achieve the best growth inhibition cells need to accumulate DNA damage induced by CP-4055 before Doc blocks the cell division. This shows that the schedule at which the combination will be given *in vivo* is important for the outcome.

In the combinations with PMX the best combination was the one in which both separate drugs had an effect on the same part of the cell cycle. It has been shown that in order to achieve a synergistic effect in the combination of gemcitabine and PMX the sequence of incubation is very important (44). In the latter study it was shown that PMX upregulated the expression of dCK and the equilibrative nucleoside transporter, which are important in the action of nucleoside analogs, the transporter possibly more than dCK. Preincubation with PMX would thus make the cells more sensitive to the prodrugs by increasing uptake, but since the prodrugs are independent of nucleoside transporters this mechanism is redundant in this case, explaining the lack of synergy.

In conclusion, the combinations with oxaliplatin were synergistic in the combination studies. Although the combinations with docetaxel did not show an enhanced effect in the combination studies based on antiproliferative assays, preincubation did increase the amount of cell kill as measured by apoptosis. The positive effect of preincubation with CP-4055 shown *in vitro* was confirmed in the animal tumor models *in vivo*.

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