Investigational New Drugs (2019) 37:415–423
https://doi.org/10.1007/s10637-018-0641-6

PRECLINICAL STUDIES

Chemosensitivity of various peritoneal cancer cell lines to HIPEC and PIPAC: comparison of an experimental duplex drug to standard drug regimens in vitro

Jürgen Weinreich[1] · Florian Struller[1] · Iaroslav Sautkin[2] · Shalva Giuashvili[3] · Marc Reymond[1] · Alfred Königsrainer[1] · Timm C. Schott[4]

Received: 22 May 2018 / Accepted: 9 July 2018 / Published online: 18 July 2018

Summary

We performed an in-vitro study testing the chemosensitivity of peritoneal cancer cell lines (SW620, HCT116, MKN45, 23,132/87, OAW42) to various cytostatic drug regimens. A duplex drug, characterized by reversible linking of the antimetabolites 2′-deoxy-5-fluorouridine (5-FdU) and 3'-C-ethynylcytidine (ECyd), was compared to oxaliplatin or to cisplatin plus doxorubicin. The experiments were designed to reflect the conditions of intraperitoneal chemotherapy. CASY® (Cell Analysis System) technology was used to compare the impact of incubation temperature/duration and drug concentration on the viability of the cancer cell lines versus normal human dermal fibroblasts. Two incubation scenarios were explored: (i) hyperthermic intraperitoneal chemotherapy (HIPEC) with 1 h of incubation at 42 °C, and (ii) pressurized intraperitoneal aerosol chemotherapy (PIPAC) with several successive incubations at 37 °C. Under HIPEC conditions, oxaliplatin induced a potent temperature-dependent growth inhibition of colon cancer cells not seen with the duplex drug. Under PIPAC conditions, the duplex drug achieved the same growth inhibition at a fraction of the dose level required with oxaliplatin. Gastric and ovarian cancer cells were more sensitive to cisplatin plus doxorubicin than to the duplex drug under PIPAC conditions. The duplex drug suggests itself, notably in cases of platinum resistance, as an alternative or addition to intraperitoneal chemotherapies when platinum-based PIPAC technology is used. Using it with HIPEC technology is not recommended. Higher doses of the duplex drug will enhance growth inhibition, albeit at the cost of a severely reduced difference in chemosensitivity between tumor and normal cells. Our findings provide orientation for PIPAC-based personalized intraperitoneal chemotherapy.

Keywords Chemosensitivity · PIPAC · HIPEC · Peritoneal cancer cell lines · Platinum versus duplex drug

Introduction

Peritoneal metastasis is a relatively common pattern of tumor dissemination, has a dismal prognosis, and remains a medical challenge yet to be mastered [1]. The standard treatment in this situation is palliative intravenous chemotherapy, and this concept is indeed supported by high-evidence comparative studies in various cancer types. Few randomized controlled trials have, however, considered peritoneal metastasis as an inclusion or exclusion criterion, and even fewer have defined its regression as a main outcome criterion. What little data is available suggests that 'specialty palliative care' might be less effective, at least in cases of colorectal cancer, when applied to peritoneal than to parenchymatous (e.g. liver or lung) metastases [2].

Intraperitoneal chemotherapy has been suggested as an alternative or additional treatment of peritoneal metastasis, making it possible to vastly increase the intraperitoneal drug concentrations, the therapeutic ratio of local to systemic dose, and hence the overall chemotherapeutic efficacy [3]. Few of today's approved anticancer drugs are suitable for application sequential to, or simultaneous with, systemic chemotherapy of peritoneal metastasis. Combinations that have been used

✉ Jürgen Weinreich
juergen.weinreich@med.uni-tuebingen.de

[1] Department of General, Visceral and Transplant Surgery, University of Tübingen, Tübingen, Germany

[2] Omsk State Medical University, Omsk, Russian Federation

[3] Department of General Surgery, Aladashvili Clinic, Ivane Javakhishvili Tbilisi State University, Tbilisi, Georgia

[4] Department of Orthodontics, Centre of Dentistry, Oral Medicine and Maxillofacial Surgery, University of Tübingen, Tübingen, Germany

include 5-FU plus folic acid plus irinotecan [4], 5-FU plus folic acid plus oxaliplatin [5], or 5-FU plus folic acid plus oxaliplatin plus irinotecan [6]. In metastatic gastric cancer, different combinations with 5-FU and platinum have been used in assessing standard chemotherapy versus best supportive care, one example being epirubicin plus cisplatin plus 5-FU [7]. The standard treatment of ovarian cancer is cytoreductive surgery followed by systemic chemotherapy with paclitaxed and carboplatin [8–11].

In selected patients with peritoneal metastasis, an approach that combines complete cytoreductive surgery and 'hyperthermic intraperitoneal chemotherapy' (HIPEC) has recently been incorporated in the standard of care [12]. The strategy of HIPEC is based on preclinical investigations into the cytotoxic activities of cisplatin and other antitumor drugs both in human cell lines and in animal models [13–19]. It was shown that, through various mechanisms, hyperthermia increased the effectiveness of these drugs. For example, intraperitoneal chemotherapy at 42 °C was found to increase the cell penetration depths and cytotoxic activities (and hence the antitumor potential) of oxaliplatin, doxorubicin [20], mitomycin C, irinocetan [21], and cisplatin [22].

'Pressurized intraperitoneal aerosol chemotherapy' (PIPAC) has been developed as a more minimally invasive alternative to HIPEC [23]. It is based on a drug delivery method in which the cytotoxic drugs are applied in the form of an aerosol (using a nebulizer) straight into the abdominal cavity under pressure at 37 °C. In this way, PIPAC might in some situations result in better drug effectiveness than HIPEC [24].

Any outcomes of intraperitoneal chemotherapy, whether applied under HIPEC or under PIPAC conditions, critically depend on the cytotoxic potential of the chemotherapeutic agents used. Our strategy is to attempt maximizing the antitumor effect of single cytostatic drugs by linking them in a chemically reversible fashion. No previous studies on record have dealt with the potential of linked cytostatic drugs for intraperitoneal chemotherapy. We prepared the ground for such investigations by reversibly linking, via a natural phosphodiester bond, the standard antimetabolite 2′-deoxy-5-fluorouridine (5-FdU) and the antimetabolite 3'-C-ethynylcytidine (ECyd) [25]. The resultant duplex drug (5FdU-ECyd) both induces strong growth inhibition in a wide variety of tumor cell lines and was shown, using cell lines of gastric adenocarcinoma as an example, to greatly improve the effectiveness of treatment as compared to widely used cytostatic drug combinations [26]. Also, the viability of both platinum-sensitive and platinum-resistant ovarian cancer cells was demonstrated to decline rapidly and in a dose-dependent fashion when exposed to the 5FdU-ECyd duplex drug [27].

Thus we aimed to evaluate how favorably this duplex drug would compare to clinically established drugs with regard to its cytotoxicity exerted in vitro on five different cancer cell lines. To address this question, we designed a study that would also include oxaliplatin used on two colon cancer cell lines (SW620, HCT 116) and cisplatin plus doxorubicin used in combination on two gastric cancer cell lines (MKN-45, 23,132/87) and one ovarian cancer cell line (OAW42). Another goal was to assess the respective temperature dependence of cell growth inhibition and the differences in chemosensitivity between human cancer cell lines and non-malignant cells.

Materials and methods

Chemical substances

The following drugs and solutions were acquired for this study: oxaliplatin (Eloxatin® 200 mg; Sanovi-Aventis, Frankfurt, Germany); cisplatin (Cisplatin Teva® 100 mg; Teva, Ulm, Germany); and doxorubicin hydrochloride (DOXO-cell® 150 mg; Cell Pharm, Bad Vilbel, Germany). The duplex drug was synthesized as described previously by our study group [25]. Peritoneal dialysis solution (Physioneal 40 Clear-Flex® Glucose; Baxter, Lessines, Belgium) was included for controls. Oxaliplatin and cisplatin were stored as original solutions in a dark place at room temperature, the doxorubicin solution in a dark refrigerator at +4 °C, and stock solutions of the duplex drug at −20 °C. Mixed Physioneal solutions were aliquoted and stored at −20 °C. The stock concentrations were oxaliplatin 5 mg/ml, cisplatin 1 mg/ml, doxorubicin 2 mg/ml, and duplex drug 1 mg/ml. Required volumes were calculated in μl and pipetted to 45 ml of the appropriate complete cell culture medium. For combinations, two drugs were added in a constant ratio.

Cell culture

All cell culture solutions were obtained from the same source (Life Technologies/Gibco; Darmstadt, Germany). The platinum-sensitive [28, 29] ovarian cancer cells (OAW42) and the non-malignant cells (natural human dermal fibroblasts) were placed in humidified incubators for culture in 150-cm^2 culture flasks (Falcon, Corning, New York, USA) with DMEM (Dulbecco's Modified Eagle Medium) plus GlutaMax-I (4.5 g/l D-glucose) containing 10% FBS (fetal bovine serum), penicillin G 100 U/ml, streptomycin 100 μg/ml, and 5% CO_2 at 37 °C. Both gastric cell lines (MKN45, 23,132/87) and one of both colon cancer cell lines (SW620) were seeded and maintained in RPMI 1640 plus GlutaMax™-I culture medium supplemented with 10% FBS, penicillin G 100 U/ml, and streptomycin 100 μg/ml. The other colon cancer cells (HCT116) were placed in humidified incubators for culture in McCoy's 5a plus GlutaMax-I culture medium containing 10% FBS, penicillin G 100 U/ml, streptomycin 100 μg/ml, and 5% CO_2 at 42 °C or 37 °C (considering that

SW620 and HCT116 were tested under both HIPEC and PIPAC conditions).

In-vitro drug treatment

The cell lines were seeded in 24-well culture plates (Falcon, Corning, New York, USA) and started at 90–100% confluence. Drug concentrations for the tests were selected based on the dosages used clinically with the HIPEC approach (see Table 1). Thus oxaliplatin was used at 300 mg/m^2 and cisplatin plus doxorubicin at 75 + 15 mg per m^2 body surface area. The latter was determined with the Dubois formula whenever body height and weight were known [30]. The clinical data resulted in mean dose levels of oxaliplatin 93.7 μg/ml and cisplatin 24 μg/ml + doxorubicin 5 μg/ml for in-vitro testing.

Figure 1 illustrates the results of a preliminary test run in which the two colon cancer cell lines (SW620 and HCT 116) were exposed to various drug concentrations at 42 °C or 37 °C for just 60 min. The cells were then washed with PBS and incubated with culture medium in the absence of drugs at 37 °C for 7 days. To determine viability, control cells were allowed to grow in the presence of Physioneal or PBS instead of a drug for the same amount of time.

Figures 2, 3, 4, 5 and 6 illustrate the results for all cell lines (cancer and non-malignant) exposed to the clinically established drugs regimens and to the experimental duplex drug at appropriate concentrations. All of these experiments were performed at 37 °C with an incubation period of 7 days, the culture medium being replaced with new drugs every 2 days. On days 0, 2, 4 and 7, the cells were washed once with 1 ml of PBS, followed by trypsinization with 250 μl of trypsin-EDTA 0.05%. Trypsinization was stopped by adding 750 μl of complete medium. The cell suspension was transferred to a 1-ml polyethylene vial and was allowed to shake in an overhead rotator at low speed until cell counting; 100 μl of the suspension were diluted with 10 ml of CASYton (Schärfe System, Reutlingen, Germany) for analysis with the automated cell counter (see next paragraph).

Cell counting

A CASY® Model TT cell counter and analyzer system (Schärfe System) was employed to determine the number and viability of tumor cells in the 24-well culture plates. The system was operated in 3 × 400 μl mode, meaning that 400 μl of the diluted (1:100 in CASYton; Schärfe System) cell suspension were drawn through the aperture and the mean of three measurements was stored. The effect of the drugs was evaluated as percentage of cell growth observed on each day of cell counting compared to the initial concentration (100%). Different software programs optimized for specific cell lines were used to determine cell parameters. Spreadsheet software (Excel 2010) was used for statistical analysis, with the data presented as mean values ± standard deviations. All experiments were performed in triplicate.

Results

We employed the various cancer cell lines (two colon, two gastric, one ovarian) as a two-dimensional cell culture model to mimic peritoneal metastasis from primary tumors in the gastrointestinal cavity. Of the two colon cancer cells lines we used, the first (SW620) is derived from sites of lymph-node involvement, thus being a suitable model for metastatic cancer, while the second (HCT116) is one of the cancer cell lines representing different histological grades of colon cancer. The ovarian cancer cell line (OAW42), established from ascites of a patient with ovarian cystadenocarcinoma, is known to produce extracellular matrix and to show a defined

Table 1 Patient characteristics and drug dosage for intraperitoneal anticancer therapy using HIPEC technology with oxaliplatin

Patient	Age (yrs)	Height (cm)	Weight (kg)	BMI (kg/m^2)	Physioneal solution (l)	Oxaliplatin dose (μg/ml)
1	52	168	95	33.7	5.5	102.5
2	29	185	108	31.6	5.7	108.9
3	74	169	65	22.8	5.5	87.5
4	59	176	150	48.4	6.0	107.1
5	65	159	49	19.4	5.0	75.0
6	45	162	83	31.6	6.0	81.4
7	50	172	93	31.4	6.0	88.6
8	60	174	100	33.0	5.0	107.5
9	64	166	61	22.1	–	85.0
Mean:	55	170	89	30.4	5.6	93.7
SD:	13	8	30	8.6	0.4	12.8
Max:	74	185	150	48.4	6.0	108.9
Min:	29	159	49	19.4	5.0	75.0

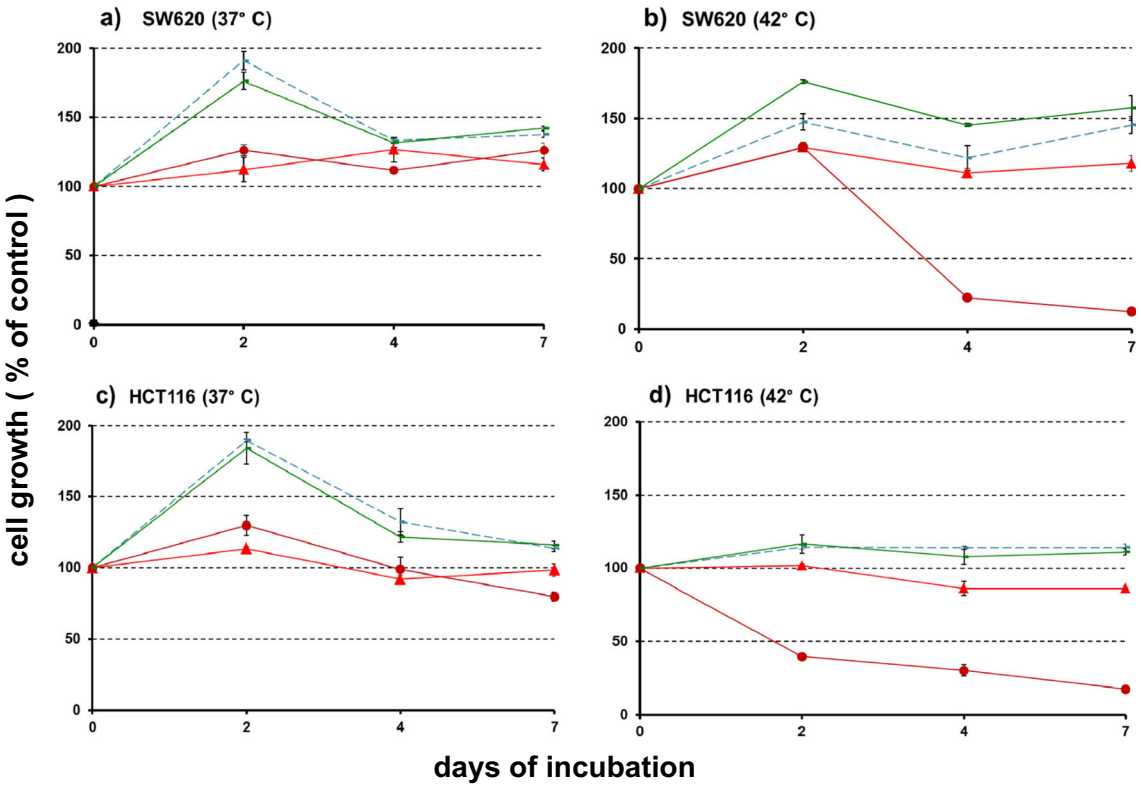

Fig. 1 Inhibition of cell growth using a 60-min drug incubation protocol. Human colon cancer cell lines (SW620, HCT116) were incubated with duplex 90 μg/ml (—▲—) or oxaliplatin 90 μg/ml (—●—) at 37 °C (**a**, **c**) or 42 °C (**b**, **d**) for 1 h, followed by incubation in culture medium without a drug for 7 days. Physioneal (- - - -) and PBS (——) controls without a drug were also used for incubation at 37 °C and 42 °C over 7 days

chemosensitivity pattern, thus being very helpful in studying the biology of human ovarian cancer.

The results described below provide orientation as to the cytotoxic potential of the duplex drug here investigated (5FdU-ECyd) for intraperitoneal chemotherapy. To determine the influence of temperature, we performed a preliminary test run in which the colon cancer cells (SW620 and HCT116) were, as an example, incubated under both PIPAC (37 °C) and HIPEC (42 °C) conditions. In clinical practice, the dose range of hyperthermically applied oxaliplatin is 85–109 mg/ml. Accordingly, we performed separate experiments incubating both colon cancer cell lines with oxaliplatin 90 μg/ml or 5FdU-ECyd 90 μg/ml at 37 °C or 42 °C for just 60 min. Each experiment was followed by washing the drug out and cultivating the cell line at 37 °C for another 7 days. Cell growth was determined using CASY® technology, which is a method widely used in numerous cytotoxicity studies [31, 32] and in monitoring cell cultivation.

Duplex drug compared to Oxaliplatin under HIPEC (42 °C) versus PIPAC (37 °C) conditions

The 60-min drug incubation protocol, with the colon cancer cell lines exposed to the duplex drug or to oxaliplatin, led to numbers of viable SW620 and HCT116 cells in a range of 90–120% (compared to 100% initially) when applied at 37 °C (Fig. 1a, c). Thus the dosages of 90 μg/ml, used for both the duplex drug and for oxaliplatin, yielded stagnating levels of cell growth without any notable reductions. The PBS control showed around 180% of cell growth on day 2 and around 130% by day 7, and a similar pattern was seen with the Physioneal control. The HCT116 cells turned out to be slightly more sensitive to both drugs than the SW620 cells. The protocol applied at 42 °C, by contrast, yielded growth reductions of both cancer cell lines to around 10% with oxaliplatin only (Fig. 1b, d). The duplex drug was associated with stagnating growth characterized by viable colon cells in the range of 90–110%, comparable to the values seen at 37 °C. The PBS control did not inhibit SW620 cell growth, while the Physioneal control did reveal a small degree of temperature-related inhibition, characterized by only a slight increase in the number of viable cells (Fig. 1b). HT116 cell growth, by contrast, stagnated at around 110% with both PBS and Physioneal at 42 °C (Fig. 1d).

Implications for the subsequent tests

All subsequent in-vitro tests were conducted at 37 °C, since (i) no temperature-related increases of cytotoxicity were seen with the

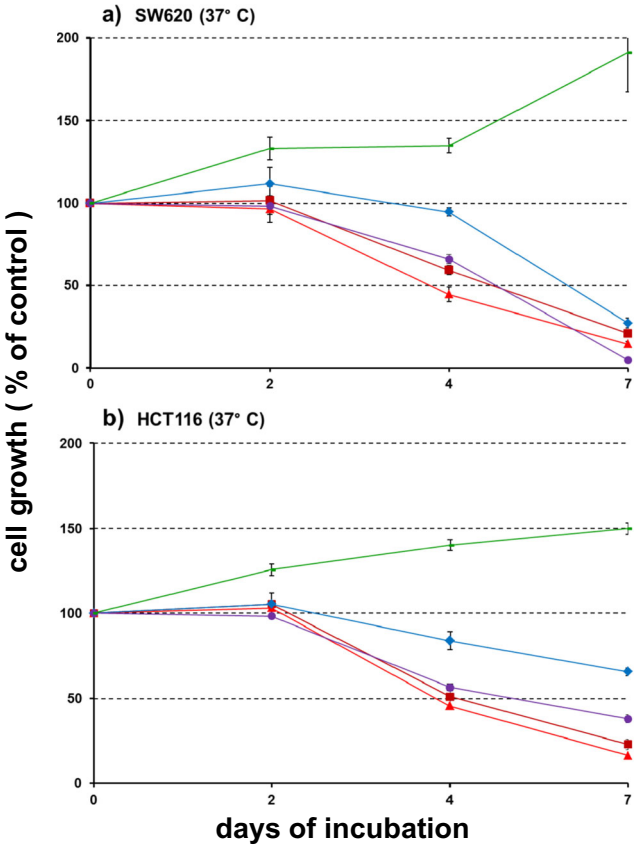

Fig. 2 Growth inhibition of human colon cancer cell lines (**a**) SW620 and (**b**) HCT116. Incubation at 37 °C was carried on over 7 days, using duplex 2 μg/ml (—▲—), duplex 1 μg/ml (—■—), oxaliplatin 16 μg/ml (—◆—), oxaliplatin 32 μg/ml (—●—), or PBS control (——)

duplex drug here under study at 42 °C and (ii) hyperthermia cannot be effectively utilized under PIPAC conditions. In the ensuing tests, the various cell lines were incubated with doses in the range of 0.32–32 μg/ml to compare the duplex drug, oxaliplatin, and the cisplatin-plus-doxorubicin combination for their cytotoxic activities at 37 °C. Lower dosages were used than in the foregoing experiments, since the incubation period at 37 °C was now 7 days, and the drug was replaced anew every second day, whereas in the aforementioned setting involving the 42 °C environment, the drug was removed from the medium after just 60 min.

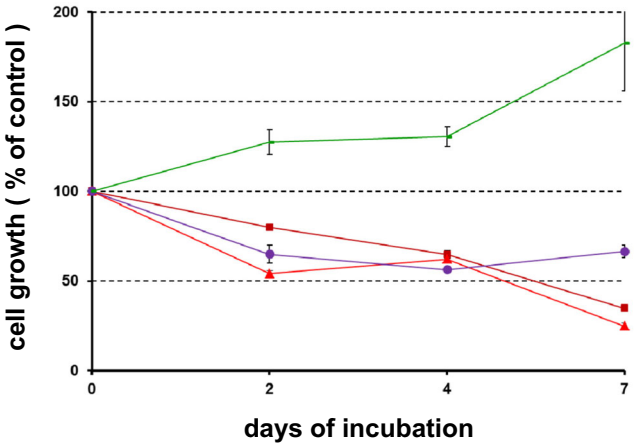

Fig. 3 Growth inhibition of non-malignant cells (normal human dermal fibroblasts). Incubation at 37 °C was carried on over 7 days, using duplex 2 μg/ml (—▲—), duplex drug 1 μg/ml (—■—), oxaliplatin 32 μg/ml (—●—), or PBS control (——)

Cell sensitivities to the duplex drug versus the standard drugs

With oxaliplatin 1 to 8 μg/ml, stagnating growth patterns of the colon cancer cell lines were found over 7 days of incubation (data not shown). SW620 growth decreased to 25% at 16 μg/ml and dropped sharply to <5% at 32 μg/ml (Fig. 2a). HCT116 growth decreased even with oxaliplatin 8 μg/ml to around 75% (data not shown) but did not fall below around 40% even at the maximum dose of 32 μg/ml (Fig. 2b). Thus the SW620 cells were more sensitive to oxaliplatin than the HCT116 cells. No such dose-dependent differences were seen with the duplex drug: 1 or 2 μg/ml would reduce SW620 and HCT116 growth to almost 20%. Duplex 2 μg/ml was slightly more effective than oxaliplatin 32 μg/ml on HCT116 cells, while the sensitivity of SW620 cells was inversely proportional at these dose levels, with oxaliplatin being more cytotoxic than the duplex drug. On balance, therefore, the duplex drug inhibited the growth of colon cancer cell lines to a similar degree as oxaliplatin at one-fifteenth of the dose level.

Colon cancer cell lines are considerably more sensitive to the duplex drug than to oxaliplatin at 37 °C when an extended incubation period is used and the drug is replaced repeatedly. For example, a one-off addition of the duplex drug at 90 μg/ml inhibited the growth of both colon cell lines only marginally at 37 °C with 1 h of incubation (see Fig. 1a, c), whereas adding the duplex drug three times at only 1 mg/ml (days 0, 2, 4) reduced this growth to 15–20% over 7 days of incubation. (Replacing the drug at the same concentration actually increases the concentration per viable cell, as dead cells and debris are removed every time the medium is replaced; only the reduced number of viable cells will be present when the new drug dose is added.) Not so with oxaliplatin, where 32 μg/ml added three times (days 0, 2, 4) at 37 °C over the 7 days of incubation yielded very much the same growth inhibition as the one-off 90 μg/ml variant with incubation at 42 °C for 1 h (see Fig. 1b, d). Thus, unlike the duplex drug, oxaliplatin did not benefit from extended incubation at lower doses with repeated replacement.

With a view to the clinical application of a cytostatic drug, one goal to keep in mind is that tumor cells should respond more sensitively than normal cells. For this reason, we also tested the duplex drug and oxaliplatin for their respective effects on a non-malignant cell line (normal human dermal fibroblasts) versus the colon cancer cell lines (Fig. 3). Incubation of the non-malignant cells with the duplex drug at 1 or 2 μg/ml caused their growth to

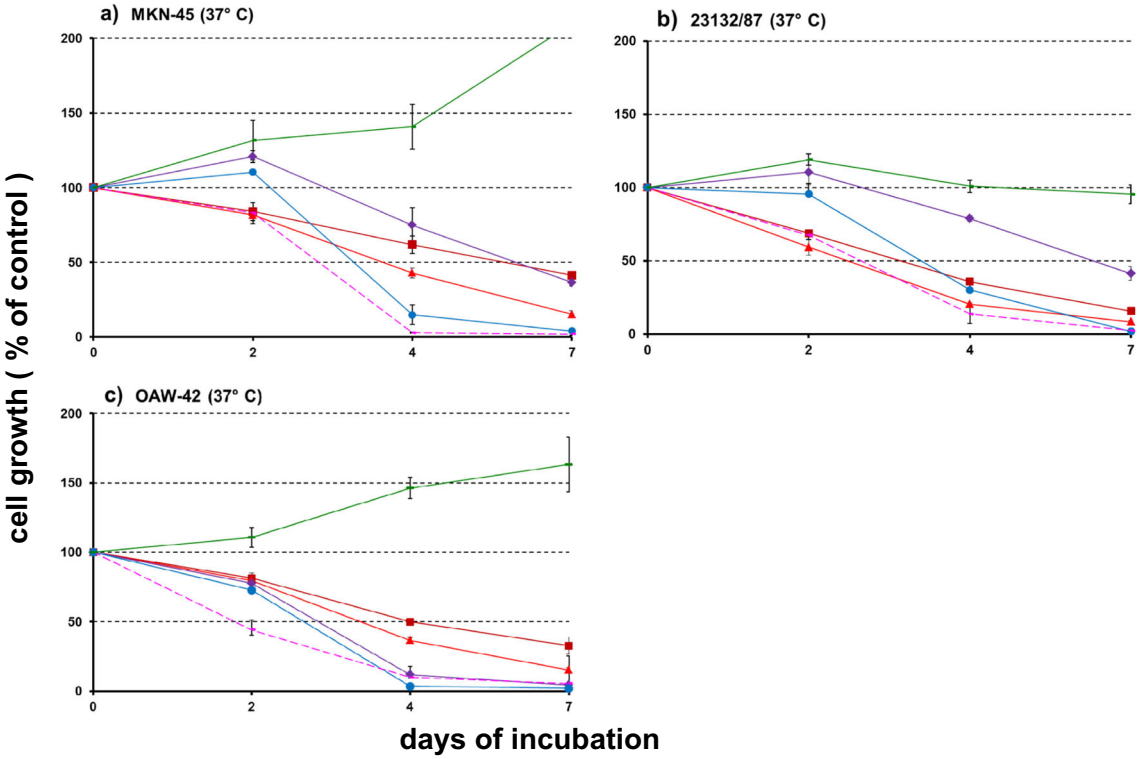

Fig. 4 Growth inhibition of human gastric cancer cell lines (**a**) MKN45 and (**b**) 23,132/87, and ovarian cancer cell line (**c**) OAW42. Incubation at 37 °C was carried on over 7 days, using duplex 2 μg/ml (—▲—), duplex 1 μg/ml (—■—), cisplatin 2.0 μg/ml + doxorubicin 1.2 μg/ml (—●—), cisplatin 1.0 μg/ml + doxorubicin 0.6 μg/ml (—◆—), cisplatin 8.0 μg/ml + doxorubicin 4.6 μg/ml (- - - -), or PBS control (——)

drop to around 25–35%. In the same situation, the colon cancer cell lines had been reduced only slightly more to around 20% (see Fig. 2). Oxaliplatin, by contrast, only reduced the non-malignant cells to around 65% but the HCT116 cells to around 40% (see Fig. 2b) and the SW620 cells to <5% when administered at 32 μg/ml (see Fig. 2a). Thus, with the duplex drug at 1–2 μg/ml, only a marginal difference in growth inhibition was seen between cancer cell lines and non-malignant cells.

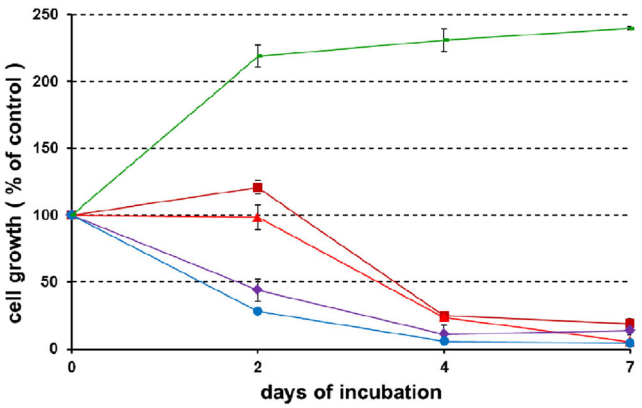

Fig. 5 Growth inhibition of non-malignant cells (normal human dermal fibroblasts). Incubation at 37 °C was carried on over 7 days, using duplex 2 μg/ml (—▲—), duplex 1 μg/ml (—■—), cisplatin 2.0 μg/ml + doxorubicin 1.2 μg/ml (—●—), cisplatin 1.0 μg/ml + doxorubicin 0.6 μg/ml (—◆—), or PBS control (——)

Like the colon cancer cells, the gastric cancer cell lines (MKN45, 23,132/87) and the ovarian cancer cell line (OAW42) differed greatly in sensitivity to the same cytostatic drugs. The duplex drug, administered at 1 or 2 μg/ml, reduced MKN45 growth to around 40% or 15%, respectively, over 7 days of incubation (Fig. 4a). Cisplatin plus doxorubicin eliminated the growth of these cells almost completely by day 7 (at 2.0 + 1.2 μg/ml) or even by day 4 (at 8.0 + 4.6 μg/ml). The 23,132/87 cells, too, were inhibited by cisplatin plus doxorubicin in a dose-dependent fashion (Fig. 4b). Slight differences between the gastric cancer cell lines were seen at the same dose levels of cisplatin plus doxorubicin, with greater sensitivity of the MKN45 than the 23,132/87 cells. Both cell lines were affected less by the duplex drug, which, at 2.0 μg/ml, inhibited their growth to around 10–20% by day 7, with slightly greater sensitivity of the 23,132/87 than the MKN45 cells. Thus, compared to cisplatin plus doxorubicin, the duplex drug entailed a reversed sensitivity differential between both gastric cancer cell lines.

Compared to these gastric cells, the ovarian cancer cell line (OAW42) was found to be more sensitive to cisplatin plus doxorubicin (Fig. 4c). This combination, applied at 2.0 + 1.2 μg/ml, reduced OAW42 growth to almost zero by day 4, whereas 2 μg/ml of the duplex drug only achieved a reduction to around 15% by day 7. OAW42 showed a sensitivity to cisplatin plus doxorubicin not too different from the non-malignant cell line (Fig. 5).

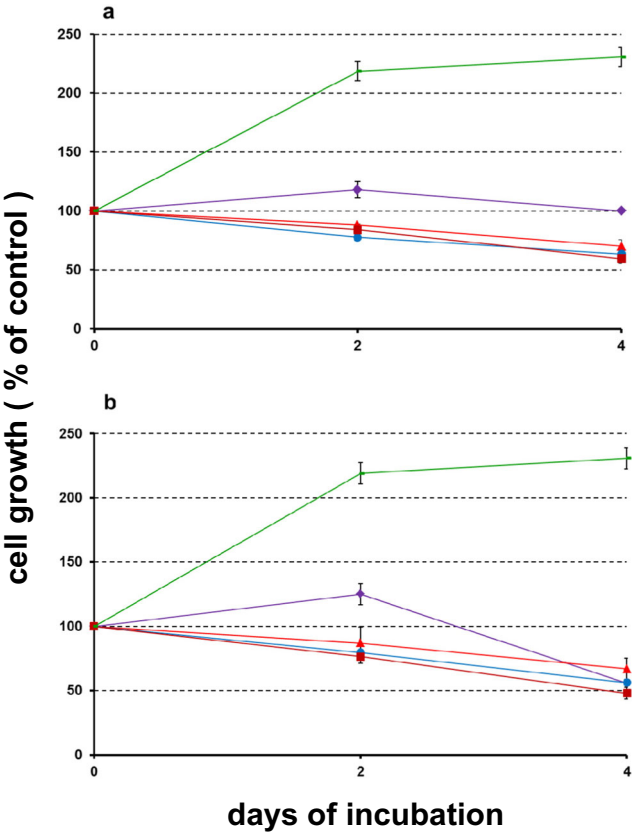

Fig. 6 Growth inhibition of MKN45 gastric (—▲—) or 23,132/87 gastric (—■—) or OAW42 ovarian cancer cell line (—●—), normal human dermal fibroblasts (—◆—), or PBS control (▬) over 4 days of incubation at 37 °C with duplex (**a**) 0.32 μg/ml or (**b**) 0.50 μg/ml

Compared to the duplex drug administered at 2 μg/ml, which reduced OAW42 cells to around 35% (Fig. 4c) and non-malignant cells even to around 25% (Fig. 5) by day 4, cisplatin plus doxorubicin administered at 2.0 + 1.2 μg/ml achieved corresponding reductions to around 5% (non-malignant cells) and 2% (OAW42). Both the non-malignant cells and the ovarian cancer cell line turned out distinctly more sensitive to cisplatin plus doxorubicin than the gastric cancer cell lines. For example, 1.0 + 0.6 μg/ml of this combination reduced both MKN45 and 23,132/87 cells to only approximately 75–80% by day 4 (Fig. 4a, b) while achieving corresponding reductions to around 10% among both the OAW42 cells (Fig. 4c) and the non-malignant cells (Fig. 5).

Figure 6 illustrates an example of how the sensitivity of cells clearly depends on the dose of the cytostatic drugs administered. For instance, the duplex drug applied at 0.32 μg/ml caused the non-malignant cells to stagnate while reducing the MKN45, 23,132/87, and OAW42 cells to between 60 and 75% by day 4 (Fig. 6a). A dose of 0.5 μg/ml, by comparison, inhibited even the non-malignant cells to a similar extent as the various gastric and ovarian cancer cells (Abb. 6b). In other words, this dose level inhibited the growth of non-malignant cells (reduction to around 55%) very much like the growth of ovarian cancer cells (reduction to 55%) and, indeed, more effectively than the growth of MKN45 gastric cancer cells (reduction to 70%).

Discussion

Current regimens of intraperitoneal chemotherapy lack in both standardization and predictability, being largely based on data extrapolated from systemic chemotherapy. After three decades of extensive research, drugs for intraperitoneal chemotherapy are today still not chosen in an evidence-based fashion. Hence there is a pressing need to generate data on chemosensitivity and chemoresistance in peritoneal metastasis, so that the large number of HIPEC protocols used in clinical practice can be standardized [33]. Our in-vitro study contributes such data by elucidating the chemosensitivity of colorectal, gastric, and ovarian cancer cell lines to a new candidate for intraperitoneal use, namely the duplex drug 5FdU-ECyd, as compared to standard antitumor drugs under HIPEC and PIPAC conditions.

Our approach of linking 5FdU to ECyd via a natural phosphodiester bond to form a duplex drug has a twofold rationale. For one thing, both the nucleobase 5FU and the nucleoside 5FdU have, along with other cytostatic drugs, yielded very successful results in polychemotherapy regimens for many years. For another, the high cytotoxic activity which the novel antimetabolite ECyd is known to exert against a multitude of diverse tumor cell lines is a good starting point for linking to 5FdU. The resultant duplex drug 5FdU-ECyd may be expected to release during its enzymatic cleavage a mixture of cytotoxic metabolites that, through various mechanisms, can have additive or synergistic activity. Hence this duplex drug, which is monotherapeutically applied on its own, constitutes a prodrug containing a mixture of various cytotoxic compounds.

Whether during parenteral or oral administration, complex transport mechanisms are required for the active substance to reach its destination. Therefore, any potential in-vivo effects cannot be predicted with complete accuracy from in-vitro data, given that transport phenomena and distribution pathways are essential to the anticancer effects of cytotoxic drugs. Any drugs used for intraperitoneal chemotherapy are applied either by allowing them to circulate inside the peritoneal cavity in a rinsing solution at 42 °C for just 60 min (HIPEC) or by pre-spraying under pressure at 37 °C in several treatment cycles (PIPAC). In this process, effective contact between the drug and the tumor cells is established. In-vitro systems, too, involve direct contact between drugs and cells, thus closely reflecting the clinical reality when it comes to evaluating the cytotoxic potential of a drug in HIPEC- or PIPAC-based intraperitoneal chemotherapy.

This study evaluated the in-vitro cytotoxic activity of the duplex drug in comparison with oxaliplatin and cisplatin plus doxorubicin, the two most common choices for intraperitoneal chemotherapy. A preliminary test run was performed under

both PIPAC (37 °C) and HIPEC (42 °C) conditions. While oxaliplatin induced a potent temperature-dependent growth inhibition of the colon cancer cell lines tested in this run, no such effect was observed with the duplex drug at 42 °C. The PBS control demonstrated that hyperthermia alone, while capable of stagnating the growth of one colon cancer cell line (HCT116), still failed to reduce this growth. Hence our results indicate that the duplex drug, rather than suggesting itself as an alternative to oxaliplatin, is not an option for use with the HIPEC technique, which takes place at 42 °C for just 60 min.

As one possible explanation for the distinct temperature-dependent effect of oxaliplatin, tumor cells might specifically take its active substance up more efficiently at 42 °C. On the other hand, uptake of the hydrophilic duplex drug 5FdU-ECyd is clearly encumbered by its polar phosphodiester bond. Also, while any of the cytostatic single drugs are immediately active, 5FdU-ECyd is an inactive prodrug that requires enzymatic cleavage to be metabolized into a mixture of cytotoxic metabolites. A higher temperature might result in partial inactivation of the metabolizing enzymes, such that the concentration of cytotoxic metabolites thus formed may be inadequately low. Enzymatic metabolization of 5FdU-ECyd by phosphodiesterase gives rise to a combination of 5FdU-5′-monophosphate, ECyd-5′-monophosphate, 5FdU, and ECyd.

The first phosphorylation of the antimetabolites sets the stage for their cytotoxic activity and is especially important if resistance against antimetabolites occurs. For example, we previously demonstrated in vitro a dose-dependent decline of ovarian cancer cell viability in response to the duplex drug, accompanied by formation of DNA double-strand breaks and by apoptosis induction. Also, the duplex drug reduced migration of the ovarian cancer cells, inhibited clonogenic or spheroidal growth, and influenced cellular pathways, DNA damage response, and RNA metabolism [27]. One hour of incubation is presumably not enough to metabolize 5FdU-ECyd in sufficient amounts, so that protracted incubation is required for 5FdU-ECyd to develop its high cytotoxicity, whereas oxaliplatin can become active immediately upon its cell uptake.

Compared to the dose level of 5FdU-ECyd, around 30 times more oxaliplatin was required in this study to reduce the growth of colon cancer cell lines to under 20% at 37 °C. At the same time, however, this strong effect of the duplex drug also affected the non-malignant cells (normal human dermal fibroblasts), leaving just a slight difference in sensitivity between normal and tumor cells. Oxaliplatin, despite its weaker cytotoxic effect, had the advantage of inhibiting the growth of non-malignant cells less than the growth of colon cancer cell lines. It is an important requirement for any novel cytotoxic drug to have a greater impact on tumor than on normal cells. Low-dose applications can make a great difference to cell sensitivity, as exemplified by clear-cut differences in sensitivity between normal and tumor cells being canceled at higher-dose applications of the same drugs. Overall, the gastric and ovarian cancer cell lines, as well as non-malignant normal cells, were clearly more sensitive to the cisplatin-doxorubicin combination than to the duplex drug 5FdU-ECyd in this study.

Several assumptions may be drawn from the results of the present in-vitro study. By linking single drugs, novel conjugates can be obtained that suggest themselves as alternative agents or supplements whenever intraperitoneal chemotherapy is required in the common event of platinum resistance. Furthermore, combining 5FdU-ECyd with standard antitumor drugs can presumably contribute to intraperitoneal chemotherapy. Thermolabile drugs are not an option for reducing tumor cell growth if intraperitoneal chemotherapy is provided, for example, in a single session using HIPEC technology at 42 °C. During a multiple-session approach under PIPAC conditions at 37 °C, advantage can be taken of the benefits offered by sequential low-dose administration of drugs. Cytotoxic efficacy will depend not only on the cytotoxic potential of specific drugs but also on the dose-dependent chemosensitivity of specific cells. Testing chemosensitivity on the basis of cell lines allows the response to drugs to be assessed in vitro, the ultimate goal being to guide decision-making for intraperitoneal chemotherapy in patients with peritoneal metastasis from gastrointestinal primary tumors.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Lambert LA (2015) Looking up: recent advances in understanding and treating peritoneal carcinomatosis. CA Cancer J Clin 65(4): 284–298. https://doi.org/10.3322/caac.21277
2. Franko J, Shi Q, Meyers JP, Maughan TS, Adams RA, Seymour MT, Saltz L, Punt CJA, Koopman M, Tournigand C, Tebbutt NC, Diaz-Rubio E, Souglakos J, Falcone A, Chibaudel B, Heinemann V, Moen J, De Gramont A, Sargent DJ, Grothey A, Analysis, research in cancers of the digestive system G (2016) Prognosis of patients with peritoneal metastatic colorectal cancer given systemic therapy: an analysis of individual patient data from prospective randomised trials from the Analysis and Research in Cancers of the Digestive System (ARCAD) database. Lancet Oncol 17(12): 1709–1719. https://doi.org/10.1016/S1470-2045(16)30500-9
3. Ceelen WP, Flessner MF (2010) Intraperitoneal therapy for peritoneal tumors: biophysics and clinical evidence. Nat Rev Clin Oncol 7(2):108–115. https://doi.org/10.1038/nrclinonc.2009.217
4. Jiang Y, Fan H, Jiang Y, Song G, Wang F, Li X, Li G (2017) Efficacy and safety of FOLFIRI and biotherapy versus FOLFIRI alone for metastatic colorectal cancer patients: a meta-analysis. Medicine (Baltimore) 96(48):e8767. https://doi.org/10.1097/MD.0000000000008767

5. Ji WB, Hong KD, Kim JS, Joung SY, Um JW, Min BW (2018) Effect of a shortened duration of FOLFOX chemotherapy on the survival rate of patients with stage II and III colon cancer. Chemotherapy 63(1):8–12. https://doi.org/10.1159/000481566
6. Marques RP, Duarte GS, Sterrantino C, Pais HL, Quintela A, Martins AP, Costa J (2017) Triplet (FOLFOXIRI) versus doublet (FOLFOX or FOLFIRI) backbone chemotherapy as first-line treatment of metastatic colorectal cancer: a systematic review and meta-analysis. Crit Rev Oncol Hematol 118:54–62. https://doi.org/10.1016/j.critrevonc.2017.08.006
7. Kang BW, Kim TW, Lee JL, Ryu MH, Chang HM, Yu CS, Kim JC, Kim JH, Kang YK, Lee JS (2009) Bevacizumab plus FOLFIRI or FOLFOX as third-line or later treatment in patients with metastatic colorectal cancer after failure of 5-fluorouracil, irinotecan, and oxaliplatin: a retrospective analysis. Med Oncol 26(1):32–37. https://doi.org/10.1007/s12032-008-9077-8
8. Bristow RE, Tomacruz RS, Armstrong DK, Trimble EL, Montz FJ (2002) Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. J Clin Oncol 20(5):1248–1259. https://doi.org/10.1200/JCO.2002.20.5.1248
9. Griffiths CT, Fuller AF (1978) Intensive surgical and chemotherapeutic management of advanced ovarian cancer. Surg Clin North Am 58(1):131–142
10. Harter P, Muallem ZM, Buhrmann C, Lorenz D, Kaub C, Hils R, Kommoss S, Heitz F, Traut A, du Bois A (2011) Impact of a structured quality management program on surgical outcome in primary advanced ovarian cancer. Gynecol Oncol 121(3):615–619. https://doi.org/10.1016/j.ygyno.2011.02.014
11. Stuart GC, Kitchener H, Bacon M, duBois A, Friedlander M, Ledermann J, Marth C, Thigpen T, Trimble E, Participants of 4th ovarian Cancer consensus C, Gynecologic Cancer I (2011) 2010 Gynecologic Cancer InterGroup (GCIG) consensus statement on clinical trials in ovarian cancer: report from the Fourth Ovarian Cancer Consensus Conference. Int J Gynecol Cancer 21(4):750–755. https://doi.org/10.1097/IGC.0b013e31821b2568
12. Eveno C, Pocard M (2016) Randomized controlled trials evaluating cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) in prevention and therapy of peritoneal metastasis: a systematic review. Pleura and Peritoneum 1(4):169–182
13. Akaboshi M, Tanaka Y, Kawai K, Akuta K, Masunaga S, Ono K (1994) Effect of hyperthermia on the number of platinum atoms binding to DNA of HeLa cells treated with 195mPt-radiolabelled cis-diaminedichloroplatinum(II). Int J Radiat Biol 66(2):215–220
14. Alberts DS, Peng YM, Chen HS, Moon TE, Cetas TC, Hoeschele JD (1980) Therapeutic synergism of hyperthermia-cis-platinum in a mouse tumor model. J Natl Cancer Inst 65(2):455–461
15. Hahn GM (1979) Potential for therapy of drugs and hyperthermia. Cancer Res 39(6 Pt 2):2264–2268
16. Herman TS, Teicher BA, Cathcart KN, Kaufmann ME, Lee JB, Lee MH (1988) Effect of hyperthermia on cis-diamminedichloroplatinum(II) (rhodamine 123)2[tetrachloroplatinum(II)] in a human squamous cell carcinoma line and a cis-diamminedichloroplatinum(II)-resistant subline. Cancer Res 48(18):5101–5105
17. Los G, van Vugt MJ, Pinedo HM (1994) Response of peritoneal solid tumours after intraperitoneal chemohyperthermia treatment with cisplatin or carboplatin. Br J Cancer 69(2):235–241
18. Meyn RE, Corry PM, Fletcher SE, Demetriades M (1980) Thermal enhancement of DNA damage in mammalian cells treated with cis-diamminedichloroplatinum(II). Cancer Res 40(4):1136–1139
19. van de Vaart PJ, van der Vange N, Zoetmulder FA, van Goethem AR, van Tellingen O, ten Bokkel Huinink WW, Beijnen JH, Bartelink H, Begg AC (1998) Intraperitoneal cisplatin with regional hyperthermia in advanced ovarian cancer: pharmacokinetics and cisplatin-DNA adduct formation in patients and ovarian cancer cell lines. Eur J Cancer 34(1):148–154
20. Istomin YP, Zhavrid EA, Alexandrova EN, Sergeyeva OP, Petrovich SV (2008) Dose enhancement effect of anticaner drugs associated with increased temperature in vitro. Exp Oncol 30(1):56–59
21. Le Page S, Kwiatkowski F, Paulin C, Mohamed F, Pezet D, Chipponi J, Benhamed M, Gilly FN, Glehen O (2006) In vitro thermochemotherapy of colon cancer cell lines with irinotecan alone and combined with mitomycin C. Hepatogastroenterology 53(71):693–697
22. Sukovas A, Cesna V, Jasukaitiene A, Barauskas G, Nadisauskiene RJ, Dambrauskas Z, Paskauskas S, Gulbinas A (2017) Response of OVCAR-3 cells to cisplatin and hyperthermia: does hyperthermia really matter? Anticancer Res 37(9):5011–5018. https://doi.org/10.21873/anticanres.11915
23. Girshally R, Demtroder C, Albayrak N, Zieren J, Tempfer C, Reymond MA (2016) Pressurized intraperitoneal aerosol chemotherapy (PIPAC) as a neoadjuvant therapy before cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. World J Surg Oncol 14(1):253. https://doi.org/10.1186/s12957-016-1008-0
24. Dueckelmann AM, Fink D, Harter P, Heinzelmann V, Marth C, Mueller M, Reinthaller A, Tamussino K, Wimberger P, Sehouli J (2018) The use of PIPAC (pressurized intraperitoneal aerosol chemotherapy) in gynecological oncology: a statement by the German "Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR)", the Swiss and Austrian AGO, and the north-eastern German Society of Gynaecologic Oncology. Arch Gynecol Obstet 297(4):837–846. https://doi.org/10.1007/s00404-018-4673-0
25. Schott H, Schott S, Schwendener RA (2009) Synthesis and in vitro activities of new anticancer duplex drugs linking 2′-deoxy-5-fluorouridine (5-FdU) with 3'-C-ethynylcytidine (ECyd) via a phosphodiester bonding. Bioorg Med Chem 17(19):6824–6831. https://doi.org/10.1016/j.bmc.2009.08.033
26. Weinreich J, Schott S, Königsrainer I, Zieker D, Königsrainer A, Schott H (2011) Cytostatic activity of the duplex drug linking 2′-deoxy-5-fluorouridine (5FdU) with 3'-C-ethynylcytidine (ECyd) against gastric adenocarcinoma cell lines. Investig New Drugs 29(6):1294–1302. https://doi.org/10.1007/s10637-010-9483-6
27. Schott S, Wimberger P, Klink B, Grutzmann K, Puppe J, Wauer US, Klotz DM, Schrock E, Kuhlmann JD (2017) The conjugated antimetabolite 5-FdU-ECyd and its cellular and molecular effects on platinum-sensitive vs. -resistant ovarian cancer cells in vitro. Oncotarget 8(44):76935–76948. https://doi.org/10.18632/oncotarget.20260
28. Beaufort CM, Helmijr JC, Piskorz AM, Hoogstraat M, Ruigrok-Ritstier K, Besselink N, Murtaza M, van IWF, Heine AA, Smid M, Koudijs MJ, Brenton JD, Berns EM, Helleman J (2014) Ovarian cancer cell line panel (OCCP): clinical importance of in vitro morphological subtypes. PLoS One 9(9):e103988. https://doi.org/10.1371/journal.pone.0103988
29. Lincet H, Guével B, Pineau C, Allouche S, Lemoisson E, Poulain L, Gauduchon P (2012) Comparative 2D-DIGE proteomic analysis of ovarian carcinoma cells: toward a reorientation of biosynthesis pathways associated with acquired platinum resistance. J Proteome 75(4):1157–1169. https://doi.org/10.1016/j.jprot.2011.10.030
30. Dubois D, Dubois EF (1916) A formula to estimate the approximate surface area if height and weight be known. Arch Intern Med 17:863–871
31. Schmut O, Faulborn J, Trummer G (1999) Quantifying the damage to conjunctival and corneal cell cultures caused by UV light using CASY (cell analysis system). A method for reducing animal experiments. Ophthalmologe 96(6):375–381
32. Winkelmeier P, Glauner B, Lindl T (1993) Quantification of cytotoxicity by cell volume and cell proliferation. ATLA 21:269–280
33. de Bree E, Michelakis D, Stamatiou D, Romanos J, Zoras O (2017) Pharmacological principles of intraperitoneal and bidirectional chemotherapy. Pleura and Peritoneum 2(2):47–62