Exposure–response relationships for oxaliplatin-treated colon cancer cells

Mark N. Kirstein[a], Stephanie A. Root[a], Megan M. Moore[a], Katie M. Wieman[a], Brent W. Williams[a], Pamala A. Jacobson[a], Paul H. Marker[b] and Todd M. Tuttle[c]

Data are lacking for an optimal infusion length for oxaliplatin administered intraperitoneally. Our objectives were to establish the roles of hyperthermia and an effective length of oxaliplatin treatment in maximizing antitumor activity. SW620 cells were treated for 0.5 vs. 2 h and at 37 vs. 42°C. Cytotoxicity, cell cycle analysis, subG$_1$ and survival were assessed with the MTT assay, flow cytometry and the clonogenic assay. The IC$_{50}$ for cells treated at 37°C was 2.90 ± 0.83 μg/ml and at 42°C, 1.99 ± 0.66 μg/ml (P = 0.14). The E_{max} for 37°C was 93.9 ± 2.57% and for 42°C, 97.8 ± 1.59% (P = 0.05). The subG$_1$ fraction did not differ between cells treated at 37 and 42°C (P = 0.12). The IC$_{50}$ for the cells treated for 0.5 h was 10.6 ± 0.60 μg/ml and for 2 h, 2.80 ± 1.70 μg/ml (P = 0.02). The E_{max} for 0.5 h was 87.9 ± 5.13% and for 2 h, 96.6 ± 3.35% (P = 0.09). SubG$_1$ for 0.5 h was 8.24 ± 1.33% and for 2 h, 15.8 ± 2.45% (P = 0.02). Clonogenic assays demonstrated diminished survival when treated with low concentrations (10 μg/ml) of oxaliplatin combined with heat treatment (P = 0.017) for 2 h, but not 0.5 h. Similar clonogenic assay experiments were performed with the oxaliplatin-resistant WiDr cell line, and differences in survival following oxaliplatin and heat treatment were again observed for 2 h, but not for 0.5 h (P = 0.002). Drug treatment for 2 h of both SW620 and WiDr cell lines is superior to treatment for 0.5 h. Cell kill effects are reliant on treatment length; hence, the choice of time exposure must be made with a view to maintaining a balance between the cell kill effects and the clinical feasibility of treating the patient. ***Anti-Cancer Drugs*** **19:37–44 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Experimental and Clinical Pharmacology, College of Pharmacy, [b]Department of Medicine and Stem Cell Institute, and [c]Department of Surgery and Cancer Center, University of Minnesota, Minneapolis, USA

Correspondence to Mark N. Kirstein, Department of Experimental and Clinical Pharmacology, College of Pharmacy and Comprehensive Cancer Center, University of Minnesota, 308 Harvard St SE, Minneapolis, MN 55455, USA
Tel: +1 612 624 5689; fax: +1 612 625 3927;
e-mail: kirst002@umn.edu

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Introduction

Oxaliplatin (*cis*-[(1*R*,2*R*)-1,2-cyclohexandiamine-*N*,*N*′] oxalate(2-)-*O*,*O*′) platinum; LOHP; Eloxatin, Bedford, Ohio, USA, a third-generation platinum complex, has been approved for use in the treatment of advanced stage colorectal cancer in combination with 5′-fluorouracil and leucovorin [1]. It has a broad spectrum of activity against colorectal, ovarian, lung and breast tumors, as well as cisplatin-resistant cell lines [2]. Oxaliplatin is non-nephrotoxic and is associated with less hematologic toxicity than other platinum agents [3]. Acute and chronic sensory peripheral neuropathies are dose limiting, and occur in over 65% of patients [4].

Oxaliplatin reacts with DNA, forming cross-links, primarily with adjacent intrastrand guanines and adenines; interstrand cross-links constitute another 5% of the adducts. Both types of adducts can result in the blockade of DNA replication and transcription. All three of the approved platinum agents create similar types of cross-links; however, adduct formation, mismatch repair status, and glutathione and glutathione-related enzyme activity were more highly correlated with cytotoxicity from cisplatin than oxaliplatin treatment in six colon cancer cell lines that were tested [5,6]. It is hypothesized that the large hydrophobic carrier ligand ring present on the molecule inhibits the mismatch repair complex, contributing to higher cell kill relative to cisplatin [7]. It has also been recently demonstrated that the nucleus is not the only target for oxaliplatin; however, these sites are still to be identified. The combination of adduct formation, inability to repair DNA and the role of non-nuclear targets can trigger the apoptotic cascade, in which BAX translocates to the mitochondria, and is followed by the release of cytochrome *c* and activation of caspase-3 [8,9].

The pharmacologic activity of oxaliplatin is influenced by several variables. After an intravenous infusion, oxaliplatin undergoes biotransformation to form up to 18 different metabolites, mainly through the nucleophilic substitution of the oxalate moiety [10]. Approximately 90–95% of the oxaliplatin found in the blood is protein bound: ultrafilterable (unbound) platinum is assumed to

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represent the entire spectrum of the platinum species with antitumor and toxic properties in circulation [11,12]. Although other platinum agents are also highly protein bound in plasma, tissue penetration by ultrafilterable oxaliplatin is over 30-fold higher than that by cisplatin or carboplatin as estimated by volume of distribution (reviewed in Ref. [10]). Clearance of the unbound form ranges from 1.5 to 382.6 l/h; it is decreased in patients with mild-to-moderate renal dysfunction, but not in those with hepatic dysfunction [4,11,13,14]. The half-life for the unbound form in human plasma is approximately 24 h. Although it has not been fully characterized yet, oxaliplatin has been shown to enter the cells through copper transporters, namely ATP7A [15,16], and organic cation transporters [17]. Genetic polymorphisms in key genes such as the excision repair cross-complementation group 1 (ERCC1) and glutathione S-transferase might also affect the pharmacology of oxaliplatin [18,19].

Investigators have attempted to overcome chemotherapy resistance, and to maximize tumor exposure and minimize systemic toxicity through direct infusion to the tumor site. Cisplatin, 5′-fluorouracil, mitoxantrone, paclitaxel and oxaliplatin have all been administered intraperitoneally (i.p.) [20–22]. Several of these compounds including oxaliplatin have also been given i.p. under hyperthermic conditions [20,23,24]. For platinum agents, the heated temperature (42°C) is hypothesized to potentiate the platinum–DNA reaction rate [25,26]. Earlier studies have established the maximum tolerated dose for oxaliplatin administered i.p. as 460 mg/m^2 when treated for 30 min under thermal conditions [24]. Although this short instillation time is practical from a clinical standpoint, a longer exposure time might be needed to achieve greater tumor kill. In these studies, we sought to establish oxaliplatin exposure–response relationships with the SW620 and WiDr cell lines. We tested oxaliplatin cytotoxicity under heated conditions to establish the role of thermal treatment of tumor cells. We have also tested the relationship between drug treatment length and cytotoxicity.

Methods

Cell culture

All cell culture operations were carried out in a sterile class II biological safety cabinet (Sterilgard III Advance; Baker Company, Sanford, Maine, USA). The SW620 and WiDr cell lines were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, Virginia, USA) containing 10% fetal bovine serum (Biosource; Rockville, Maryland, USA), 2 mmol/l of glutamine, 50 units/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen, Carlsbad, California, USA). The cells were grown in 25- and 75-ml flasks (Corning, New York, USA) in a humidified incubator at 37°C with 5% CO_2 (Forma Scientific, Marietta, Ohio, USA). Cells were stained with Trypan blue (Sigma-Aldrich, St Louis, Missouri, USA) and then counted on a hemacytometer.

Monolayer growth assays

To assess the sensitivity of the cells to oxaliplatin (Sanofi-Synthelabo, New York, New York, USA), growth assays were carried out using MTT (Sigma-Aldrich). Using 24-well plates (Corning), SW620 or WiDr cells were seeded in triplicate at a density of 15 000 cells/well. After adhering overnight, the SW620 cells were treated with oxaliplatin concentrations ranging from 0.1 to 1000 μg/ml or with drug-free media for 0.5 or 2 h at either 37 or 42°C. Drug-containing medium was removed and the wells were washed with a drug-free medium. Fresh, drug-free medium was then added to the wells and the cells were incubated for 3 days at 37°C. The WiDr cells were treated with oxaliplatin concentrations ranging from 0.1 to 1000 μg/ml or with drug-free media for 2 h at 37°C, followed by similar incubation conditions in drug-free media for 3 days. At the termination of the waiting period, each well was treated with 0.17 mg/ml of MTT in 1 × phosphate-buffered saline (PBS) for 4 h in a humidified incubator at 37°C in 5% CO_2. Afterwards, the MTT solution was removed and the cells were treated with isopropanol 99 + %, of spectrophotometric grade (Acros Organics USA, Morris Planes, New Jersey, USA). The absorbance was measured by a spectrophotometer set at 550 nm. The triplicate values were averaged and the background absorbance was subtracted. The fraction for each concentration, relative to control, was calculated. The Hill equation (see below) [27] was fitted to the data using the maximum-likelihood estimation as implemented in Adapt II [28]. Model parameters that were estimated included maximum effect (E_{max}), concentration to inhibit 50% of cell growth (IC_{50}) and the slope of the curve (γ). The maximum effect is defined as the measure of cell kill that can occur at the highest drug concentrations tested. This value is useful to assess the relative cytotoxicity that is possible at concentrations similar to those achieved in clinical studies (i.e. 0.15–0.23 mg/ml). At least three independent experiments were carried out to test the relationship between length of drug exposure and cytotoxicity at 37°C, and to test treatment at two different temperatures, 37 and 42°C.

$$\text{Absorbance} = 1 - \left(E_{max} \times C^{\gamma} / IC_{50} + C^{\gamma} \right).$$

Flow cytometry

For cell cycle analysis of SW620 cells grown in static culture, 100-mm^2 plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) were seeded with 500 000 cells and allowed to adhere overnight. On the following day, the cultures were treated with 300 μg/ml of oxaliplatin or with drug-free media as control for 0.5 or 2 h at 37°C (three treated plates at each treatment time and one control). This concentration approximates the peritoneal

instillate concentrations of oxaliplatin that were measured in an earlier pharmacokinetic study [24]. In separate experiments, plate cultures were treated with 300 μg/ml of oxaliplatin or with drug-free media for 2 h at either 37 or 42°C. The medium was then removed, the culture washed once with drug-free media, fresh drug-free medium added and the plates incubated for 24 or 72 h at 37°C. Cells were trypsinized, harvested and counted with a hemocytometer.

Immediately after harvest, cells were treated with 1% paraformaldehyde in PBS for 15 min at 4°C. Thereafter, cells were stored at −20°C in PBS/ethanol. On the day of flow cytometry, the cells were prepared with propidium iodide for cell cycle analysis as described previously [29]. On the day of analysis, the cells were reconstituted in 1 ml of 3.8 mmol/l of sodium citrate containing 50 μg/ml of propidium iodide (Sigma-Aldrich) and 125 μg/ml of RNAse A (Worthington Biochemical, Lakewood, New Jersey, USA). Briefly, the cells were analyzed on a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, California, USA) flow cytometer gated on forward light scatter pulse height and side scatter pulse height for the analysis of cell cycle fractions, and in the ungated mode for the detection of cells with subG_1 DNA content. The histograms were then evaluated with Flow Jo Watson Pragmatic v. 6 software (Tree Star, Ashland, Oregon, USA). Cell cycle and apoptotic fractions were compared between treatment groups with the Student's two-sample *t*-test.

Colony-forming assay

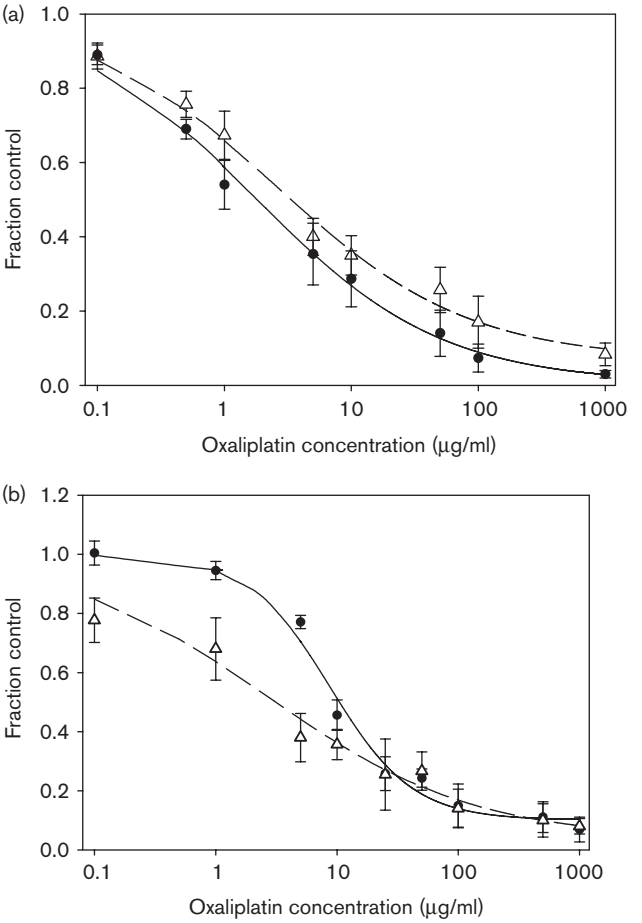
The clonogenic assay was used to assess survival [30]. SW620 and WiDr cells (range 1×10^5–8×10^5) were seeded in duplicate in 25-ml tissue culture flasks and incubated overnight in a humidified incubator at 37°C with 5% CO_2. At 24 h after seeding, the cells were treated with 10 μg/ml of oxaliplatin or with drug-free media as control. The cells were treated for either 0.5 or 2 h and at either 37 or 42°C in a humidified incubator with 5% CO_2. After treatment, the cells were washed with PBS, and fresh drug-free medium was added. Flasks were incubated in a humidified incubator at 37°C with 5% CO_2 for 14 days. The colonies were washed with PBS, fixed with 5 ml of a 10 : 1 methanol/acetic acid solution for 15 min, and stained with 5 ml of 1% crystal violet in 10 : 1 methanol/acetic acid. Colonies $\geq$ 1 mm were counted, and the survival fraction (ratio of number of colonies survived to number of colonies plated) was calculated. All values were normalized to their respective controls. Five independent experiments were performed and the results were compared with the *t*-test.

Results

Treatment temperature and cytotoxicity

The effect of hyperthermia on oxaliplatin cytotoxicity was tested for the SW620 cells. The cells were treated for 2 h at concentrations ranging from 0.1 to 1000 μg/ml at either 37 or 42°C. Afterward, the cells were incubated in drug-free media at 37°C for 3 days and then analyzed by the MTT assay. Shown in Fig. 1a are the cytoxicity curves for SW620 cells treated at the two temperatures. The IC_{50} for the cells treated at 37°C was 2.90 ± 0.83 μg/ml and at 42°C, 1.99 ± 0.66 μg/ml ($P = 0.14$). The E_{max} for the cells treated at 37°C was 93.9 ± 2.57% and at 42°C, 97.8 ± 1.59% ($P = 0.05$).

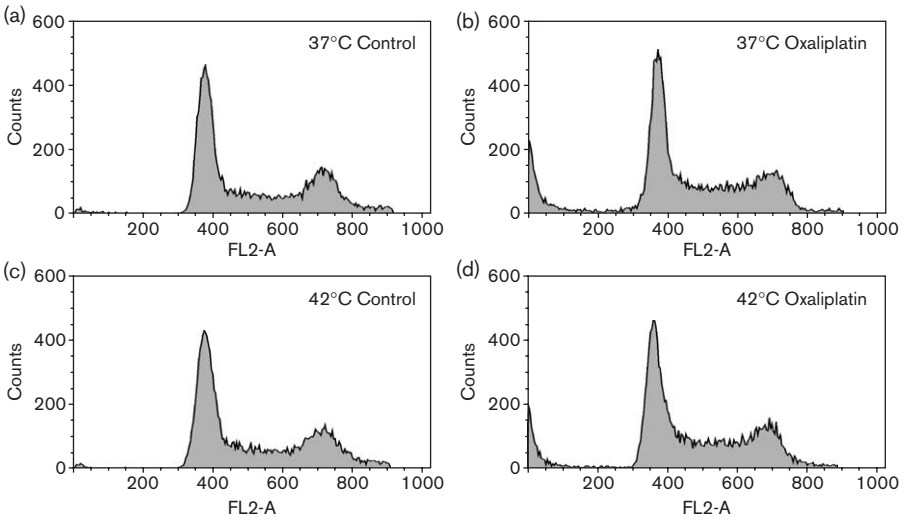
Fig. 1



Effects of thermal treatment on sensitivity to oxaliplatin and of time length of oxaliplatin exposure on SW620 cell death. (a) Dose–response (i.e. fraction control vs. log oxaliplatin concentrations) curves for SW620 cells after treatment with oxaliplatin for 2 h at 37°C (–Δ–) or 42°C (–●–), followed by incubation in drug-free media for 72 h at 37°C. (b) Dose–response curves for SW620 cells after treatment with oxaliplatin for 0.5 h (–●–) or 2 h (–Δ–), followed by incubation in drug-free media at 37°C for 72 h. Concentrations were tested in triplicate and in three independent experiments. Points, mean; bars, ± SD.

To further test the effects of thermal treatment on cell kill, SW620 cells were treated with 300 μg/ml of oxaliplatin and incubated for 2 h at 37 or 42°C. After the treatment, the cells were incubated in drug-free media for 72 h and analyzed by flow cytometry (Fig. 2).

Fig. 2



Effects of thermal treatment on sensitivity to oxaliplatin. Representative flow histograms of SW620 cells recovered from separate 100-mm plates and treated with propidium iodide. Histogram (a) SW620 cells treated with drug-free media for 2 h at 37°C as control, followed by incubation in drug-free media at 37°C for 72 h, (b) SW620 cells after treatment with 300 μg/ml of oxaliplatin for 2 h at 37°C, followed by incubation in drug-free media at 37°C for 72 h, (c) SW620 cells treated with drug-free media for 2 h at 42°C as control, followed by incubation at 37°C in drug-free media for 72 h, (d) SW620 cells after treatment with 300 μg/ml of oxaliplatin for 2 h at 42°C, followed by incubation at 37°C in drug-free media for 72 h.

Table 1 Cell cycle analysis (% mean ± SD) for SW620 cells treated with 300 μg/ml of oxaliplatin or drug-free media as control at either 37 or 42°C for 2 h (three independent experiments)

Treatment	G_1	S	G_2/M	SubG_1
37°C Control	43.2 ± 4.87	35.5 ± 8.97	19.8 ± 3.66	1.49 ± 0.61
42°C Control	40.0 ± 1.89	38.1 ± 2.41	20.4 ± 1.25	1.49 ± 0.25
t-test				$P = 0.99$
37°C Oxaliplatin	33.3 ± 1.48	40.4 ± 1.16	12.6 ± 1.66	13.8 ± 1.59
42°C Oxaliplatin	32.2 ± 3.42	44.1 ± 3.33	13.5 ± 1.78	10.2 ± 2.43
t-test				$P = 0.12$

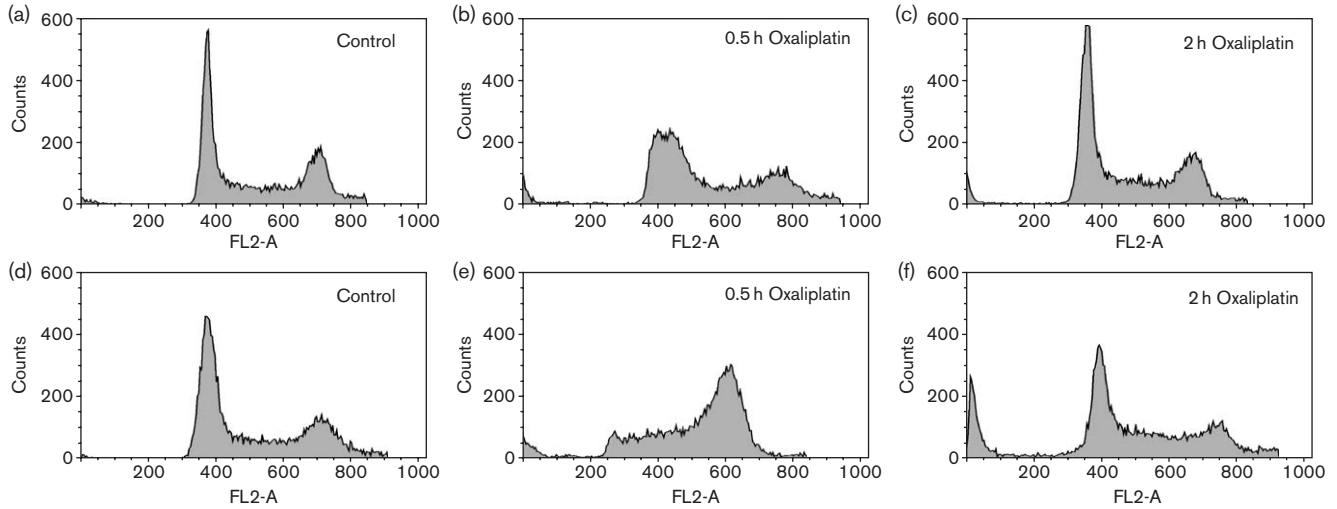
The data for cell cycle and subG_1 are presented in Table 1. Relative to the 37°C control, a significant decrease was observed for the G_2/M fraction ($P = 0.05$) and a nine-fold increase for the subG_1 fraction. The two sets of controls did not differ from each other with respect to subG_1, suggesting that thermal treatment alone does not affect cell death.

Treatment length and cytotoxicity

We wanted to evaluate whether there was a difference in cytotoxicity between the 0.5- and 2-h treatment times. The 0.5-h treatment was chosen on the basis of the results of previous clinical studies and the clinical practicality of a short i.p. treatment time [24]. The longer treatment time was limited to 2 h as it is not feasible to keep human patients under anesthesia for a longer period of time, especially after surgical debulking. After the cells were incubated with the drug for 0.5 or 2 h at 37°C, cells were incubated in drug-free media for an additional 72 h, and this was followed by cytotoxicity analysis. Shown in Fig. 1b are the cytotoxicity curves for SW620 cells treated with oxaliplatin for 0.5 and 2 h. The IC_{50} for the cells treated for 0.5 h was 10.6 ± 0.60 μg/ml and for those treated for 2 h, it was 2.80 ± 1.70 μg/ml ($P = 0.02$). The E_{max} for the cells treated for 0.5 h was 87.9 ± 5.13% and for those treated for 2 h, it was 96.6 ± 3.35% ($P = 0.09$). The slope of the dose–response curve for the cells treated for 0.5 h was consistently steeper than that for cells treated for 2 h ($P < 0.001$), accounting for the statistical significance for IC_{50} and not E_{max}. Therefore, the measurable difference in cytotoxicity between the two treatment times tends to differ at concentrations that are lower than those achieved in i.p. infusions (0.15–0.23 mg/ml).

To further test the effects of time on cell kill, SW620 cells were treated with 300 μg/ml of oxaliplatin and incubated for 0.5 vs. 2 h. The drug concentration that we tested closely approximates the drug concentrations that are expected in the peritoneal fluid following a peritoneal infusion [24]. After treatment, the cells were incubated in drug-free media for 24 or 72 h, and analyzed by flow cytometry (Fig. 3). The data for cell cycle and subG_1 are presented in Table 2. By 24 h, the subG_1 had increased relative to control, but was not different between the 0.5- and 2-h treatment groups. S-phase arrest was observed for the 0.5-h but not for the 2-h samples. By 72 h, the percentage of subG_1 cells had increased further, and was higher for cells that had been treated for 2 h compared with those treated for 0.5 h. In contrast to 2-h-treated samples, cells treated for 0.5 h showed a significant

Fig. 3



Effects of time length of oxaliplatin exposure at 37°C and also of the posttreatment incubation time on SW620 cell death as measured by the subG$_1$ fraction. Representative flow histograms of the SW620 cells recovered from separate 100-mm plates and treated with propidium iodide. Histogram (a) SW620 cells treated with drug-free media as control, followed by incubation in drug-free media for 24 h, (b) SW620 cells after treatment with 300 μg/ml of oxaliplatin for 0.5 h, followed by incubation in drug-free media for 24 h, (c) SW620 cells treated with 300 μg/ml of oxaliplatin for 2 h, followed by incubation in drug-free media for 24 h. Histogram (d) SW620 cells treated with drug-free media as control, followed by incubation in drug-free media for 72 h, (e) SW620 cells after treatment with 300 μg/ml of oxaliplatin for 0.5 h, followed by incubation in drug-free media for 72 h, (f) SW620 cells treated with 300 μg/ml of oxaliplatin for 2 h, followed by incubation in drug-free media for 72 h.

Table 2 Cell cycle analysis (% mean ± SD) for SW620 cells treated with 300 μg/ml of oxaliplatin or drug-free media as control for either 0.5 or 2 h, followed by incubation in drug-free media for either 24 or 72 h (three independent experiments)

Treatment	G_1	S	G_2/M	SubG_1
Control	45.9 ± 5.96	32.7 ± 10.2	21.0 ± 4.31	1.19 ± 0.49
24 h Harvest				
0.5 h Oxaliplatin	24.5 ± 2.36	54.1 ± 4.39	16.6 ± 1.72	5.52 ± 1.05
2 h Oxaliplatin	40.0 ± 3.50	39.2 ± 2.80	15.8 ± 0.88	6.94 ± 1.74
t-test	0.006	0.02	0.26	0.31
72 h Harvest				
0.5 h Oxaliplatin	2.90 ± 0.23	48.4 ± 1.27	40.4 ± 2.16	8.24 ± 1.33
2 h Oxaliplatin	27.5 ± 2.29	46.2 ± 1.63	10.6 ± 1.12	15.8 ± 2.45
t-test	0.003	0.13	0.0002	0.02

increase in the percentage of cells arrested at the G_2/M phase.

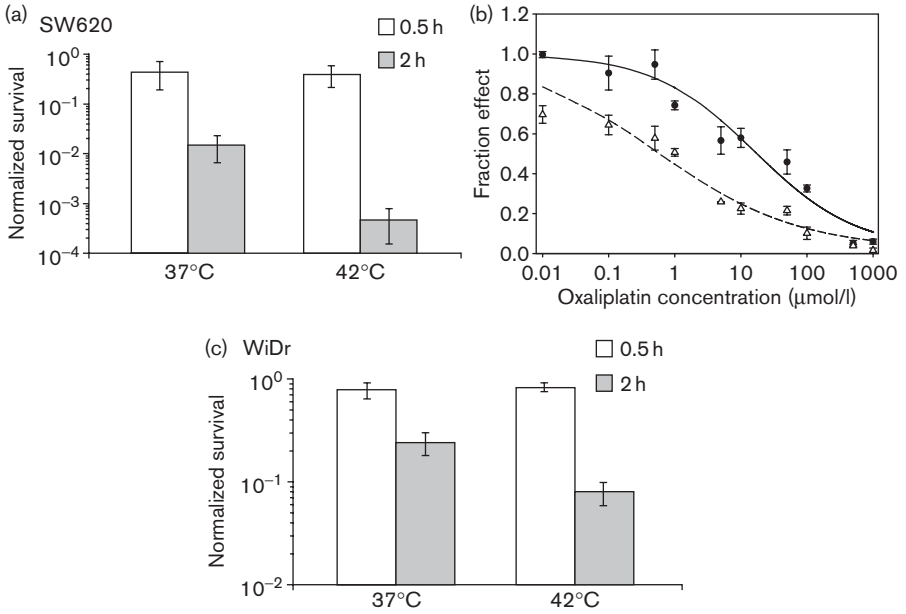
Colony formation

As the cells in the 0.5-h group were predominantly in the G_2/M phase, and those in the 2-h group in the G_1/S phase, we conducted colony-forming experiments to assess long-term survival (i.e. beyond 72 h). We initially conducted experiments using oxaliplatin at 100 and 300 μg/ml, respectively; however, we did not observe a sufficient number of colonies after the treatment. Therefore, we tested with oxaliplatin at 10 μg/ml. Clonogenic survival after treatment with oxaliplatin or with drug-free medium is shown in Fig. 4a. Survival figures after control treatment at 37 and 42°C were 48 and 52%, respectively. Survival figures (normalized to respective controls) after oxaliplatin treatment for 0.5 h at 37 and 42°C were 44.9 and 37.8%, respectively ($P = 0.63$). Survival figures after oxaliplatin treatment for 2 h at 37 and 42°C were 1.48 and 0.046%, respectively ($P = 0.017$). A lower survival rate was observed after the 2-h treatment than after the 0.5-h treatment at 37°C ($P = 0.02$). We tested for a cell line that was more resistant to oxaliplatin treatment than SW620 (Fig. 4b); after three independent experiments, we estimated that the IC_{50} for WiDr cells was over 30 times that of SW620 cells. To test whether time and temperature are also additive for resistant cells, we performed similar colony-forming assay experiments with WiDr cells after treatment with 10 μg/ml of oxaliplatin (Fig. 4c). Survival rates after control treatment at 37 and 42°C were 24.7 and 24.9%, respectively. Survival rates (normalized to respective controls) after oxaliplatin treatment for 0.5 h at 37 and 42°C were 78.1 and 82.5%, respectively ($P = 0.55$). Survival rates after oxaliplatin treatment for 2 h at 37 and 42°C were 24.2 and 7.91%, respectively ($P = 0.002$). A lower survival rate was observed after the 2-h treatment than after the 0.5-h treatment, at 37°C ($P = 0.0002$).

Discussion

Several reports describe surgical debulking, followed by the local i.p. administration of adjuvant hyperthermic chemotherapy as a new treatment modality for metastatic peritoneal carcinomas [24,25,31,32]. These operations are extensive, often last 12–14 h and are associated with significant complications. Despite resection of all gross

Fig. 4



Effects of heat (42°C) and of treatment times of oxaliplatin for SW620 and WiDr cells ($n=5$). Dose–response curves for the SW620 and WiDr cells. Colony formation was assessed as percentage survival relative to the number of plated cells 2 weeks after the drug treatment and was normalized to the respective controls. Data points are mean ± SD. (a) Bar plots depicting colony-forming assay data for SW620 cells treated with 10 μg/ml of oxaliplatin under the indicated conditions, (b) dose–response curves for SW-620 (–Δ–) and WiDr cells (–●–) treated with oxaliplatin for 2 h at 37°C, followed by incubation in drug-free media for 72 h. Concentrations were tested in triplicate and in three independent experiments, (c) bar plots depicting colony-forming assay data for WiDr cells treated with 10 μg/ml of oxaliplatin under the indicated conditions.

disease, however, these tumors inevitably recur within the abdominal cavity. To minimize recurrence, investigators have used adjuvant chemotherapy for the treatment of micrometastatic tumors. As mentioned previously, it is often given locally to maximize tumor exposure and to minimize systemic exposure and toxicity. Local administration of chemotherapy can be accomplished through the use of an inflow catheter in the upper abdomen and an outflow catheter in the lower abdomen. This process enables the drug to be reheated, whereupon it reenters the peritoneal space. Oxaliplatin is usually prepared in a 2-l/m^2 (0.15–0.23 mg/ml) solution, infused at approximately 1 l/min at 41–42°C into the abdominal cavity and held for 0.5 h. The aims of these studies were to assess the role of hyperthermia in oxaliplatin-induced cytotoxicity and to establish an effective length of time for instillation by chemotherapy, through in-vitro studies.

Here, we were able to show that exposure to 42°C heat augments the cytotoxicity of the oxaliplatin-treated SW620 and WiDr cells in the 2-h treatment group. This effect was observed at the end of a longer period of posttreatment incubation time (i.e. 2 weeks) than either the MTT assay or flow cytometry methods would allow. The colony-forming assay enables the easier evaluation of drug effects on both actively dividing cells and quiescent cells, unlike the other two cytotoxicity methods that we used. The MTT assay demonstrated a small increase in cytotoxicity related to thermal treatment, but the differences were not significant overall. We also did not see a significant increase in cell kill as measured by the subG_1 fraction. Other investigators have reported enhanced cell kill *in vitro* when cells were treated with oxaliplatin under hyperthermic conditions [33,34]. They tested the following cell lines: SW 1573 (squamous cell human lung tumor), FSa-II (mouse fibrosarcoma), and Caco-2 and HT-29 (human colon carcinoma), which were treated with oxaliplatin concentrations ranging from 7.5 to 39.7 μg/ml for durations ranging from 5 to over 150 min (60 min most common). For the colony-forming assays, we initially tested oxaliplatin concentrations that were a log-fold higher (300 μg/ml) than those tested by others. Even when seeding up to 2 million cells per flask, we were unable to obtain enough countable colonies following treatment with 100 and 300 μg/ml, respectively, for both cell lines. Therefore, we conducted our colony-forming assays with subtherapeutic concentrations of the drug (10 μg/ml). It is possible that treatment temperature becomes less relevant at therapeutic concentrations of drug as there will be few if any surviving cells after treatment with these higher concentrations.

We also demonstrate that exposure of SW620 cells to oxaliplatin for 2 h, compared with 0.5 h, shows superior

antitumor activity. Our results are in agreement with the findings of Mishima *et al.* [35], although they tested for much longer times of exposure (i.e. 1 h vs. 10–14 days). Mishima demonstrated an almost 2 log-fold decrease in the IC_{50} between cells treated for the short vs. the long time period. They also measured both the intracellular platinum and the DNA–platinum adducts formation after exposure to oxaliplatin concentrations ranging from 20 to 100 μg/ml for 1 h. Mishima and others have shown that both platinum and adduct concentrations were higher with increasing oxaliplatin concentrations, but were lower with oxaliplatin-resistant cell lines [35,36]. These results suggest the importance of both whole-cell platinum uptake and adduct formation as independent predictors of cell response to drug treatment. As mentioned previously, the cellular uptake and efflux for oxaliplatin are mediated at least in part by the ATP-dependent copper transporter, ATP7A and organic cation transporters. Therefore, prolonged exposure to oxaliplatin might enable a greater cellular uptake of platinum through this and other unknown processes.

Cell cycle analysis demonstrated that SW620 cells treated for 0.5 h accumulate in the G_2/M phase when harvested 72 h after treatment. Similar findings after this time (i.e. 72 h) have been reported by others for HT-29, $CaCO_2$, DLD1 and SW-480 colon cancer cells [9,37]. Concurrently, we observed a large G_1 fraction decrease, especially in the 0.5-h-treated samples and a small S-phase increase. To test whether 2-h oxaliplatin treatment results in an earlier increase for G_2/M than was observed at 72 h, we repeated the experiment, but harvested after only 24 h. We did not observe any significant increase of G_2/M for either the 0.5-h or the 2-h-treated samples after 24 h. We did, however, observe an increase of the S fraction in the 0.5-h samples. An increase of S-phase is possible as DNA–adduct formation would be expected to interfere with the DNA replication process. Overall, we observed that cells treated for 0.5 h accumulate first in the S-phase; this is followed later by a significant accumulation in G_2/M. Accumulation of 2-h-treated cells at any phase was less remarkable than that of the shorter-treatment cells.

In summary, we showed for the first time that the treatment of cells with clinically relevant oxaliplatin concentrations for 2 h, compared with 0.5 h, results in a greater than twofold increase in cell death. Studies with an animal model of i.p.-administered oxaliplatin will enable a better understanding of the possible clinical relevance of these results. Although others have established the maximum tolerated dosages for oxaliplatin given over 0.5-h infusions as 460 mg/m^2 [24], this would need to be reassessed if a 2-h instillation is attempted. A longer exposure time might be associated with greater toxicity, necessitating a dosage decrease. Therefore, a phase I study would be required to determine the maximum tolerated dosage under these conditions.

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