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ORIGINAL PAPER

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Hyperthermia enhances the cytotoxicity and platinum-DNA adduct formation of lobaplatin and oxaliplatin in cultured SW 1573 cells

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Abstract The cytotoxicity of cisplatin and cisplatin-DNA adduct formation in vitro and in vivo is clearly enhanced by hyperthermia. We investigated whether cytotoxicity and platinum-DNA adduct formation of two promising new third-generation platinum derivatives, lobaplatin [1,2-diamminomethylcyclobutane platinum(II) lactate] and oxaliplatin [oxalato-1,2-diaminocyclohexane platinum(II)], are also enhanced by hyperthermia. Cisplatin was used for comparison. SW 1573 cells were incubated with cisplatin, lobaplatin or oxaliplatin at different concentrations for 1 h at 37°, 41° and 43°C. The reproductive capacity of cells was determined by cloning experiments. Immunocytochemical detection of platinum-DNA adducts was performed with the rabbit antiserum NKI-A59. At 37°C, cisplatin was the most cytotoxic, followed by oxaliplatin and lobaplatin. Hyperthermia clearly enhanced the cytotoxicity of cisplatin, lobaplatin and oxaliplatin. There was no further increase in cytotoxicity at 43°C compared to 41°C for cisplatin and oxaliplatin. A further increase in cytotoxicity at 43°C was observed for lobaplatin. At 43°C thermal enhancement was higher for lobaplatin than for oxaliplatin, with the reverse pattern at 41°C. For both drugs, thermal enhancement of cytotoxicity was lower than observed for cisplatin.

Immunocytochemical detection of platinum-DNA adducts was feasible for all the drugs. Adduct formation was enhanced at 43°C for cisplatin, lobaplatin and oxaliplatin with a relative increase of 410%, 170% and 180%. These results seem to confirm that an increase in platinum-DNA adduct formation is involved in the in vitro thermal enhancement of cytotoxicity. The observed thermal enhancement of cytotoxicity of lobaplatin and oxaliplatin in vitro warrants further in vivo investigations.

Key words Hyperthermia, induced · Lobaplatin · Oxaliplatin · Cisplatin · Platinum-DNA adduct formation · Cytotoxicity

Abbreviation *TER* thermal enhancement ratio

Introduction

The cytotoxicity of cisplatin in vitro and in vivo is enhanced by hyperthermia. This thermal enhancement is maximal when hyperthermia and cisplatin are given simultaneously (Barlogie et al. 1980; Fisher and Hahn 1982; Hahn and Li 1982; Haas et al. 1984; Murthy et al. 1987; Dahl and Mella 1990; Urano et al. 1990; Los et al. 1991).

Recently, Los et al. (1993, 1994) found that hyperthermia primarily affected the cellular uptake of cisplatin. As a result of this increase in cellular uptake, cisplatin-DNA adduct formation was also increased.

Lobaplatin [1,2-diamminomethylcyclobutane platinum(II) lactate] and oxaliplatin [oxalato-1,2-diaminocyclohexane platinum(II)] are new third-generation platinum derivatives with promising antitumour activity and a different toxicity profile compared to cisplatin (Voegeli et al. 1990; Fiebig et al. 1992; Weiss and Michael 1993; Tashiro et al. 1989; Mathe et al. 1989; Kelland 1993; Levi et al. 1993). It is not yet known

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whether the cytotoxicity and platinum-DNA adduct formation of these new derivatives are also enhanced by hyperthermia. Therefore, we investigated the *in vitro* cytotoxicity (at 37°C, 41°C and 43°C) and platinum-DNA adduct formation (at 37°C and 43°C) of lobaplatin and oxaliplatin in comparison to results with cisplatin.

Materials and methods

Cells

SW 1573 cells were used, which originated from a human squamous cell lung tumour. The cells were isolated and characterized by Dr. A. Leibovitz, Temple, Tex., USA. Cells were grown routinely at 37°C in Leibovitz (L-15, Gibco) medium, supplemented with 10% fetal calf serum and 2 mM fresh glutamine. The L-15 medium does not require enhanced CO₂ pressure in the incubator. Absence of *Mycoplasma* infection was checked regularly using an enzyme-linked immunosorbent assay *Mycoplasma* detection kit (Boehringer, Mannheim, Germany). The doubling time of the cells during exponential growth was approximately 22.9 h. Cells were passaged weekly. In all experiments exponentially growing cells were used.

The reproductive capacity of the cells was determined by cloning experiments. After treatment, cells were washed twice with Hanks' balanced salts solution and detached from the surface of the culture dish by 0.05% trypsin in balanced salts solution.

After resuspension in fresh culture medium, the cells were counted using a Coulter counter model Dn (Coulter, Hertfordshire, UK), plated at multiple dilutions in cluster dishes and incubated for 8–10 days. Colonies were fixed and stained in 6% glutaraldehyde solution containing 0.25% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. The cloning efficiency of SW 1573 cells treated at 37°C for 1 h varied between 0.60 and 0.70.

Hyperthermia treatment

Exponentially growing SW 1573 cells were incubated in petri dishes (diameter 6 cm) with the different platinum compounds at different concentrations for 1 h. During this treatment the dishes were placed on grids in a thermostatically controlled ($\pm 0.05^\circ\text{C}$) waterbath. The cells were incubated for 1 h at 37°C, 41°C or 43°C in culture medium of pH 7.35. When control dishes were placed in the waterbath, they reached the desired temperature ($\pm 0.2^\circ\text{C}$) within 4 min. In order to obtain 37°C, 41°C and 43°C in the dishes the waterbath temperature had to be 37.3°C, 41.4°C and 43.4°C respectively. All experiments were performed in duplicate and repeated two or three times. The data for the combined treatment are corrected for the effect of heat alone.

Drugs

Lobaplatin was kindly provided by ASTA Medica AG, Dresden, Germany. Oxaliplatin was kindly provided by Debio S.A., Lausanne, Switzerland. Cisplatin (Platinol) was obtained from Bristol-Myers Squibb, Woerden, the Netherlands. The different concentrations of the platinum compounds were freshly prepared shortly before cell incubation for 1 h.

Thermal enhancement of each drug was defined as the ratio of the dose causing 99% cell death at 37°C to that at 41°C (TER 41) and to that at 43°C (TER 43).

Processing of cells for immunocytochemical visualization of platinum-DNA adducts

SW 1573 cell incubation with the different platinum compounds was carried out as described above. Cells were treated for 1 h at 37°C or 43°C with 10–50 μM cisplatin or 5–100 μM lobaplatin or oxaliplatin.

After two washes and trypsinization of cells, cells were centrifuged twice for 10 min at 200 *g* and resuspended in phosphate-buffered saline. Cytospins were performed 5 h after incubation for cisplatin (Los et al. 1993) and oxaliplatin and 12 h after incubation for lobaplatin. The post-incubation time in drug-free medium was, therefore, respectively 4, 4 and 11 h. These assay times were chosen as preliminary experiments had shown that maximal adduct formation at 37°C occurred at these times for the different drugs (data not shown). Cytospin preparations were made on ovalbumin-coated slides and immunocytochemically analysed for platinum-drug-induced DNA modifications. All experiments were performed in duplicate.

Immunocytochemical analysis of platinum-DNA modification

The immunoperoxidase staining procedure was carried out as described before with some modifications, as specified below (Terheggen et al. 1988).

Cytospin slides were treated with phosphate-buffered saline H₂O₂ (to inactivate endogenous peroxidases), 1 M KCl, proteinase K, ethanol/NaOH (the last three steps served to denature the DNA and to increase the accessibility of the platinum-DNA adducts), 1% bovine serum albumin (to reduce nonspecific antibody binding) and rabbit antiserum NKI-A59 raised against cisplatin-modified calf thymus DNA (Terheggen et al. 1991). To visualize the cisplatin-DNA adducts we used a biotinylated F(ab')₂ fragment of swine anti-(rabbit immunoglobulin) (Dako, Denmark), an avidin/horse-radish-peroxidase vector for amplification and 3,3'-diaminobenzidine-hydrochloride/nickel as peroxidase substrate. Each sample was stained in duplicate in separate stainings on different days. The staining intensity of individual nuclei was measured microdensitometrically with a Knott (München, Germany) light-measuring device (beam diameter 0.5 μm), coupled to a Leitz Orthoplan microscope. The scanning equipment was linked to an Atari ST computer (Atari, Sunnyvale), programmed with an adapted version of the histochemical data acquisition system (Hidacsy, Leiden, The Netherlands). The selected density, defined as the integrated absorbance of a selected area, expressed in arbitrary units, was determined on 15 nuclei/slide (Van Benthem et al. 1991). The adduct increase ratio per compound was defined as the ratio of the intensity of the nuclear stain at 43°C to that at 37°C at an equimolar concentration of 50 μM .

Results

Cytotoxicity at 37°C

All three drugs showed significant cytotoxicity in the SW 1573 cells after 1 h incubation at 37°C (Fig. 1). The iso-effect cytotoxicity at a relative survival of 50% was observed at concentrations of 15 μM for cisplatin, 12 μM for oxaliplatin and 50 μM for lobaplatin. The iso-effect cytotoxicity at a relative survival of the cells of 1% was observed at concentrations of 62.5 μM for cisplatin, 67.5 μM for oxaliplatin and 127.5 μM for lobaplatin. Iso-effect cytotoxicity at a relative survival

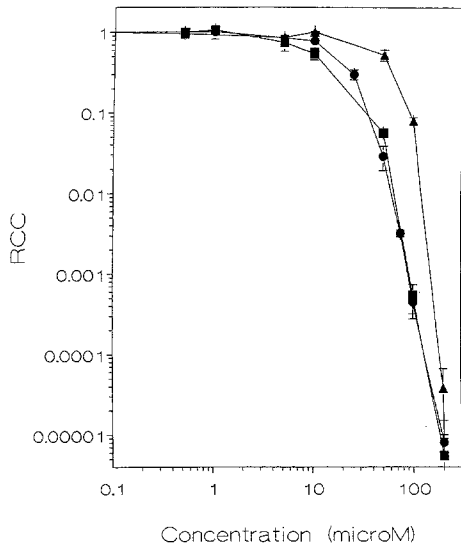


Fig. 1 Cytotoxicity of cisplatin (●), oxaliplatin (■) and lobaplatin (▲) in SW 1573 cells after 1 h incubation at different concentrations at 37°C, expressed as relative clonogenic capacity (*RCC*) with standard deviation

of 0.1% was observed at 90 μM for cisplatin, 93 μM for oxaliplatin and 157.5 μM for lobaplatin. These results show that cisplatin was most cytotoxic, followed by oxaliplatin and lobaplatin (relative toxicity 1:0.95:0.55).

Cytotoxicity at elevated temperatures of 41°C and 43°C

Hyperthermia clearly enhanced the cytotoxicity of cisplatin (Fig. 2). There was no clear further increase in

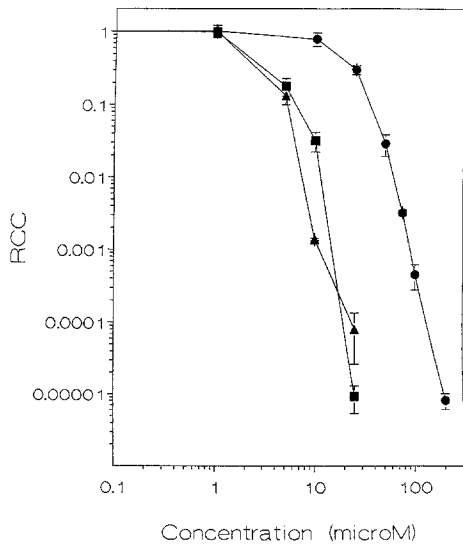


Fig. 2 Cytotoxicity of cisplatin in SW 1573 cells after 1 h incubation at 37°C (●), 41°C (■) and 43°C (▲) expressed as relative clonogenic capacity (*RCC*) with standard deviation

cytotoxicity at 43°C when compared to 41°C. The thermal enhancement ratio (TER) at the iso-effect level of 1% survival was 7.8 at 43°C (see also Table 1). Thermal enhancement of cytotoxicity was first observed at a concentration of 5 μM .

The cytotoxicity of lobaplatin was also enhanced by hyperthermia, with a further increase in cytotoxicity between 41° and 43°C (Fig. 3). The TER was 2.9 at 43°C and 1.6 at 41°C. Thermal enhancement of cytotoxicity was first observed at a concentration of 10 μM .

For oxaliplatin there was also a clear thermal enhancement from 37°C to 41°C, with no further increase between 41°C and 43°C (Fig. 4). The TER was 2.1 at

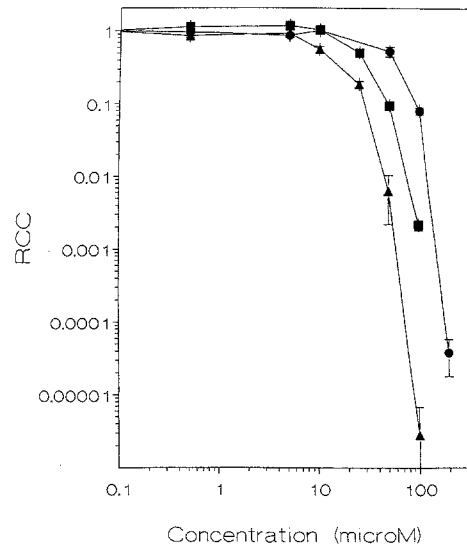


Fig. 3 Cytotoxicity of lobaplatin in SW 1573 cells after 1 h incubation at 37°C (●), 41°C (■) and 43°C (▲) expressed as relative clonogenic capacity (*RCC*) with standard deviation

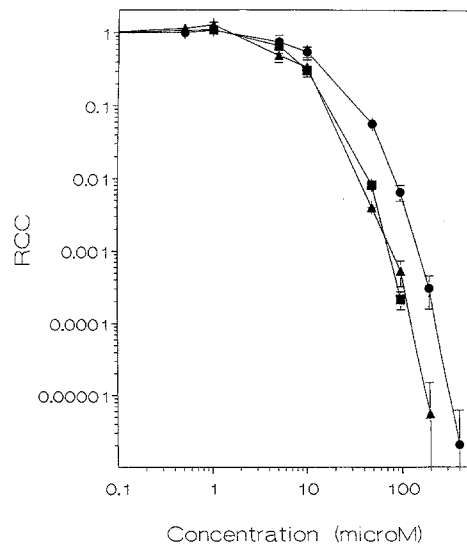


Fig. 4 Cytotoxicity of oxaliplatin in SW 1573 cells after 1 h incubation at 37°C (●), 41°C (■) and 43°C (▲) expressed as relative clonogenic capacity (*RCC*) with standard deviation

43°C and 2.0 at 41°C. Thermal enhancement of cytotoxicity was first observed at a concentration of 10 μM .

The TER at 43°C was highest for cisplatin, followed by lobaplatin and oxaliplatin. At 41°C the highest TER was observed for cisplatin, followed by oxaliplatin and lobaplatin.

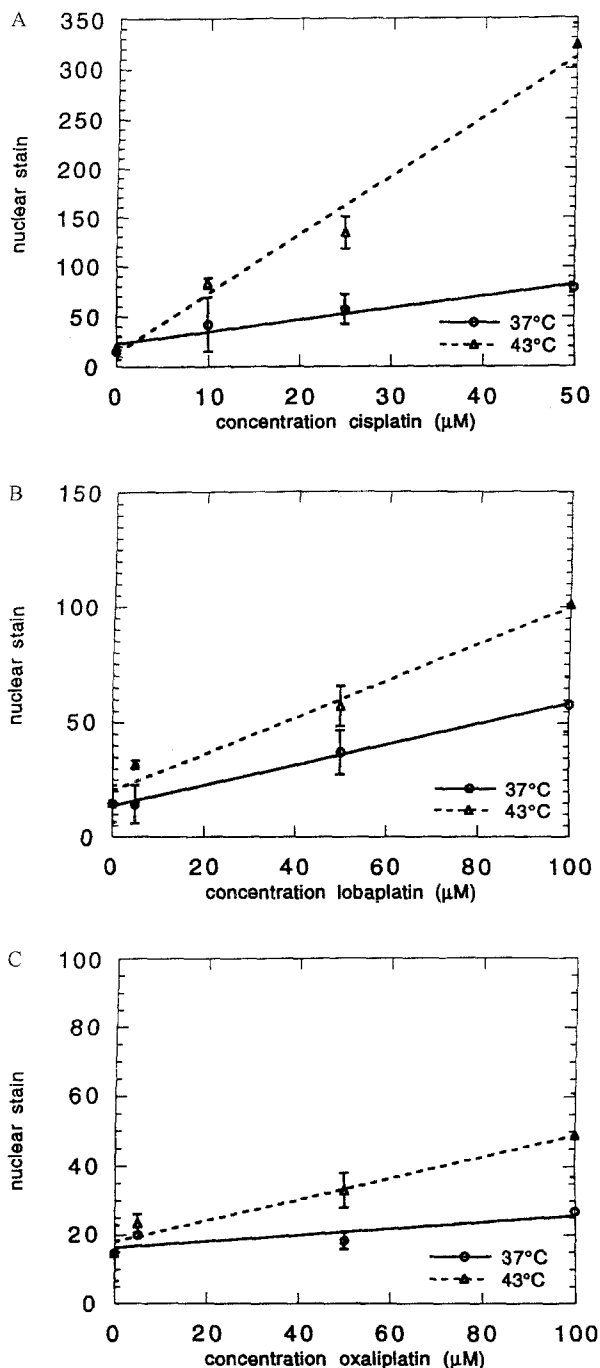


Fig. 5A–C Platinum-DNA adduct formation for cisplatin (A), lobaplatin (B) and oxaliplatin (C), expressed in arbitrary units with standard deviation at 37°C and 43°C, measured with an immunocytochemical technique. Adducts were determined 5 h after incubation for cisplatin and oxaliplatin, and 12 h after incubation for lobaplatin

Table 1 Thermal enhancement ratio at 43°C (*TER 43*) and 41°C (*TER 41*) Compared to 37°C measured at the point of 99% cell death. Adduct increase ratio (*AIR*) at 43°C compared to 37°C measured at a concentration of 50 μM

Drug	TER 41	TER 43	AIR
Cisplatin	7.3	7.8	4.1
Lobaplatin	1.6	2.9	1.7
Oxaliplatin	2.0	2.1	1.8

Platinum-DNA adduct formation at 37°C and 43°C

The results are presented in Fig. 5A–C. Platinum-DNA adduct formation, after 1 h of incubation at 37°C, expressed in arbitrary units (AU), measured at a equimolar concentration of 50 μM , showed the highest adduct level for cisplatin (78.6 ± 4.9 AU) followed by lobaplatin (37.3 ± 9.6 AU) and oxaliplatin (18.3 ± 2.5 AU). We were thus able to detect the platinum-DNA modification induced by lobaplatin and oxaliplatin with the NKI-A59 antiserum. A clear linear relation was observed between the doses given and the nuclear stain levels.

Platinum-DNA adduct formation was clearly enhanced by hyperthermia for all drugs. The adduct increase ratio at 43°C was calculated at the equimolar concentration of 50 μM and is given in Table 1. The ratio was largest for cisplatin, followed by oxaliplatin and lobaplatin. The area under the curve for the adducts and the ratio of increase at 43°C compared to 37°C were also calculated. Results were comparable to the adduct increase ratio at 43°C, with a ratio of 4.1 for cisplatin, 1.8 for oxaliplatin and 1.7 for lobaplatin.

Discussion

In this paper, we present the results of in vitro investigations of the cytotoxicity and platinum-DNA adduct formation caused by hyperthermia in combination with two new third-generation platinum derivatives, lobaplatin and oxaliplatin. Cisplatin was used as the basis for comparison, because the cytotoxicity of cisplatin and platinum-DNA adduct formation are clearly enhanced by hyperthermia (Dahl and Mella 1990; Los et al. 1993, 1994). Los et al. (1993, 1994) found that hyperthermia not only increased the killing effect but also led to a doubling in cisplatin-DNA adduct formation at 41.5°C compared to 37°C. In an intraperitoneal rat tumour model there was also a clear thermal enhancement of cisplatin cytotoxicity, as well as an increase in adduct formation. The adduct increase ratio was 2.7 (Los et al. 1994).

Our results confirm these findings for cisplatin in the SW 1573 cells at 43°C compared with 37°C. Heat not only increased cytotoxicity (TER 7.8) but also led to

a more than fourfold increase in platinum-DNA adduct formation. Whether this is the mechanism responsible for the observed thermal enhancement of cytotoxicity cannot be adequately answered. Other mechanisms, such as an inhibition of DNA repair, might be involved (Stege et al. 1995).

Of the new platinum compounds, oxaliplatin was slightly more cytotoxic at 37°C – measured at a relative survival of 0.1% – than lobaplatin at an equimolar concentration. The same pattern was observed at lower, more clinically relevant, concentrations (Stewart et al. 1982).

The cytotoxicities of both lobaplatin and oxaliplatin were clearly enhanced by hyperthermia, although the thermal enhancement ratio was smaller than the ratio observed for cisplatin. At 43°C the ratio of thermal enhancement was higher for lobaplatin than for oxaliplatin, with the reverse pattern at 41°C. This was caused by the phenomenon that the cytotoxicity of lobaplatin further increased from 41°C to 43°C, in contrast to what was observed for cisplatin and oxaliplatin (no difference between 41°C and 43°C). Thermal enhancement was already observed at relatively low drug concentrations.

Immunocytochemical detection of platinum-DNA adducts with the rabbit antiserum NKI-A59 evidently was feasible for both new drugs. This means that NKI-A59, which was originally raised against cisplatin-modified calf thymus DNA, is not only able to recognize cisplatin- and carboplatin-induced DNA modifications (Terheggen et al. 1991; Blommaert et al. 1993), but also DNA modifications induced by other platinum derivatives. Previous investigations with the antiserum NKI-A59 showed that there was a good correlation *in vitro* between the surviving fraction and the nuclear density induced by cisplatin (Terheggen et al. 1990). NKI-A59 recognizes one or more of the bifunctional intrastrand Pt-GG or Pt-AG adducts (Terheggen et al. 1988, 1990; Blommaert et al. 1993) without the option to quantify them separately. This implies that oxaliplatin and lobaplatin probably induce a DNA modification, which at least must have partial epitopic similarity to these already-known cisplatin-DNA adducts. It is likely that NKI-A59 recognizes DNA conformational alterations caused by platinum rather than the molecular structure of the adduct itself (Rice et al. 1989; Leng 1990). The staining does not give information about the absolute adduct levels of the three compounds. The higher staining level of cisplatin, compared to lobaplatin and oxaliplatin, therefore does not necessarily reflect actual differences in the adduct levels, since the affinity of the antiserum towards adducts containing various carrier ligands might be different.

Platinum-DNA adduct formation was clearly enhanced at 43°C compared to 37°C for cisplatin, lobaplatin and oxaliplatin, with a relative increase of respectively 410%, 170% and 180%. These results

seem to confirm that an increase in platinum-DNA adduct formation is involved in the *in vitro* thermal enhancement of cytotoxicity of these drugs, as has been shown by Los et al. (1993, 1994) for cisplatin. Whether the measured increase in adducts is responsible for the thermal enhancement of the cytotoxicity of oxaliplatin and lobaplatin cannot be adequately answered. This is because there are the following problems. We do not know if all the platinum-DNA adducts formed by the different platinum compounds were recognized by the NKI-A59 antiserum and, if so, whether they were measured with similar efficiency. Fichtinger-Schepman et al. (1989) have shown that immunocytochemical detection of platinum-DNA adducts is dependent on the induced degree of DNA modification, and thus on the concentration used. Whether platinum-protein actions should be taken into account or whether an altered frequency distribution of the platinum-DNA adducts should be considered under hyperthermic conditions cannot be determined from our data. However, an altered frequency distribution seems not to be very likely, because Fichtinger-Schepman et al. (1985) found no change in adduct pattern when they incubated salmon sperm DNA with cisplatin at different temperatures. Determination of the precise contribution of platinum-DNA adduct formation in the thermal enhancement of cytotoxicity of lobaplatin and oxaliplatin and absolute comparison of platinum-DNA adducts between the drugs is therefore not possible.

Further research should aim to clarify these issues by using different methods such as like atomic absorption spectroscopy, immunochemical techniques and post-labelling (Blommaert and Saris 1995). The observed thermal enhancement ratios for lobaplatin and oxaliplatin of 2.9 and 2.1 at 43°C and 1.6 and 2.0 at 41°C warrant further *in vivo* investigations.

Nowadays, with locoregional hyperthermia it is possible to reach a temperature of 41°C (Overgaard et al. 1995; van Dijk et al. 1990), while whole-body hyperthermia is capable of inducing 41.8°C (Robins et al. 1993). The combination of locoregional hyperthermia with cisplatin (Rietbroek et al. 1995, 1996) or ifosfamide (Issels et al. 1990) has proven to be feasible and has given promising response rates. The combination of whole-body hyperthermia and carboplatin at 41.8°C was also feasible, with ongoing multicentre studies (Robins et al. 1993). Cisplatin cannot be used at a systemic temperature of 41.8°C, because of severe renal toxicity (Gerad et al. 1983; Mella et al. 1987). New drugs, like lobaplatin and oxaliplatin, with limited or no cross-resistance with cisplatin and no nephrotoxicity, that *in vitro* show clear enhancement of cytotoxicity by hyperthermia, are therefore interesting for further *in vivo* studies. Only if these *in vivo* studies show clear thermal enhancement of cytotoxicity at clinically relevant dosages, together with acceptable toxicity, could further investigations in patients be considered.

Lobaplatin showed activity in patients with refractory or relapsed ovarian cancer (Gietema et al. 1995) and also had potential activity in patients with non-small-cell lung cancer and oesophageal cancer (Fiebig et al. 1992; Kelland 1993). The dose-limiting toxicity of lobaplatin was a renal-function-related thrombocytopenia; if this toxicity is influenced by hyperthermia remains to be established. Oxaliplatin showed activity in metastatic colorectal cancer (Levi et al. 1993), especially in combination with fluorouracil and leucovorin (Levi et al. 1994). There is also potential activity in oesophageal, lung and urothelial cancer (Extra et al. 1990). The dose-limiting toxicity was a peculiar sensory neuropathy, while neither renal nor haematological toxicities were encountered (Extra et al. 1990). Whether this toxicity is influenced by the combination with hyperthermia also remains to be established.

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