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Chemoresistance in Rat Ovarian Tumours

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In a cisplatin resistant subline (O-342/DPP) of an intraperitoneally growing transplantable rat ovarian tumour (O-342), intracellular glutathione (GSH) was approximately doubled (mean [S.E.] 1.5 [0.26] vs. 0.8 [0.2] nmol/10^6 cells). GSH reductase activity was higher (30.64 [4.07] vs. 20 [0.92] nmol/min per mg protein), although no difference was found for GSH-S-transferase. 24 h after exposure to cisplatin, formation of DNA interstrand cross-links was at a maximum in both lines and significantly higher in O-342 (162 [23] vs. 88 [22] rad eq). Combination treatment of O-342/DDP with buthionine sulphoximine plus cisplatin resulted in a marginal increase in survival compared with cisplatin treatment; treatment of this line with 3-aminobenzamide plus cisplatin was also superior to cisplatin alone. In the sensitive line both combinations were likewise superior to cisplatin alone. *In vitro*, at equimolar concentration, a new platinum complex (CTDP) was at least as active as cisplatin in both lines, which suggests a superior therapeutic index because its LD_{50} in mice is threefold higher than that of cisplatin. A ruthenium complex (ICR) had a higher activity in the resistant line. A titanium complex (budotitane) was not active.
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INTRODUCTION

MANAGEMENT OF ovarian cancer has been improved by the introduction of cisplatin although the development of resistance limits its use. To find new drugs that are superior to cisplatin or are active in cisplatin-resistant tumours, a series of new metal compounds was developed by one of us (B.K.K.), including complexes of platinum, titanium, zirconium, hafnium or ruthenium [1–4]. These compounds were tested in different experimental tumour models, among them autochthonous mammary and colorectal cancer in the rat, and some have shown promising results. To have an appropriate ovarian tumour model for further evaluation of these drugs and to study resistance mechanisms in experimental ovarian tumours, we are establishing a panel of such models, including animal and human tumour lines. One model, rat tumour O-342 and its cisplatin-resistant subline, O-342/DPP, is described here. Glutathione (GSH) modulation by buthionine sulphoximine and modulation of DNA repair by 3-

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aminobenzamide were studied. Also, first results with three new metal complexes are presented.

MATERIALS AND METHODS

Tumour model

Ovarian tumour O-342 was induced in a pregnant BD IX rat by intraperitoneal injection of ethylnitrosourea 100 mg/kg. The tumour was manifest in 422 days. The tumour was passaged *in vivo* by intraperitoneal inoculation of minced tumour tissue (about 5×10^6 tumour cells per animal). Resistance of O-342 to cisplatin was induced *in vivo*: after administration of seven doses of cisplatin 1.88 mg kg over about 3 weeks (passage 1), in each subsequent passage an average of 2–3 doses of cisplatin 1.5 mg kg were administered over about 24 months (about 40 passages). Stability of resistance of O-342/DDP was monitored by measuring sensitivity to cisplatin at different times. In addition, karyotyping was used to determine the stability of both lines, since in the sensitive line O-342 hyperploid clones (3n) and in the resistant line near-diploid clones predominate (G. C. *et al.*).

GSH and GSH-related enzymes

Tumour cells were washed twice in Dulbeccos's phosphate-buffered saline (PBS) and lysed by addition of double-distilled water. Cellular proteins were precipitated with 5% trichloroacetic acid. After centrifugation at 1400 *g* (10 min, 4°C), the reduced glutathione was assayed in the supernatant [5]. Although this method is not specific for GSH and detects all non-protein thiols of cells, GSH constitutes over 90% of such thiols in tumour cells [6]. Mean GSH-values of O-342 and O-342/DDP were based on the analysis of 11 and 13 tumour samples in triplicate, respectively. For assay of GSH-related enzymes 5–7 samples of each tumour line were analysed in triplicate; cell suspensions were sonicated on ice (2 min, 100 W) and subsequently centrifuged (6 min, 10000 *g*). The protein content of the supernatant was determined. Activity of GSH_2-reductase was measured according to Goldberg and Spooner [7] and activity of GSH-S-transferase according to Habig *et al.* [8].

DNA breaks and cross-links

DNA single-strand breaks and interstrand cross-links were measured by alkaline elution [9] with slight modifications: after tumour removal from the animals (10–12 days after inoculation), a single cell suspension was obtained by mincing the tumours with scissors in RPMI 1640 supplemented with 10% fetal calf serum (Sigma), 100 U/ml penicillin, 1% glutamine and 8% HEPES (Fa. Seromed, Berlin), and treatment with collagenase/dispase dissolved in Dulbecco's PBS (0.1 U/ml collagenase, 0.8 U/ml dispase) for 1 h at 25°C. The cells were filtered through a polyester mesh 0.20 mm (Reichelt Chemie Technik). Cells were exposed to cisplatin for 1 h at 37°C, followed by incubation for different times in cisplatin-free medium before alkaline elution. 1 ml cell suspension ($1.5–2.0 \times 10^6$/ml, viability over 80%) was loaded onto polycarbonate filters (Nucleopore GmbH, Tübingen). Cells were lysed with a buffer containing sodium dodecyl sulphate and proteinase K (Sigma) at pH 10. After continuous washings at pH 10, the DNA was eluted at 0.03 ml/min in alkaline buffer (pH 12.2). Ten fractions, each containing 2 ml, were collected over 66 min each. Subsequently the filters of each holder were minced and incubated in 6 ml alkaline buffer at 37°C for 2 h to force the remaining DNA into suspension. The DNA content of all 10 fractions as well as of the filter suspension was assayed fluorimetrically with Hoechst dye 33258 (Serva, Heidelberg). Further evaluation procedures have been described [10]. We used a continuous-flow analyzer (type 5100 Skalar Analytical, Breda, Netherlands) holding 375 samples (sample processor SP100) and fluorimeter (type 6300). To introduce a controlled level of DNA single-strand breaks, a specimen of cells was subjected to gamma-irradiation (500 rads = 5 Gy) in the dark at 0°C (137Cs, Gammacell 100, Atomic Energy of Canada Ltd; dose rate 16.7 Gy/min). DNA-interstrand cross-links were expressed in rad equivalents (rad eq).

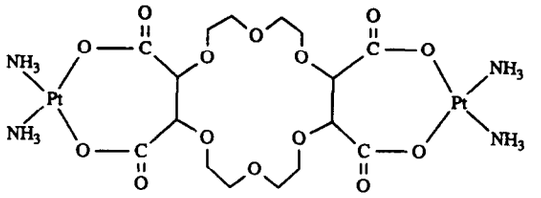

Fig. 1. Structure of CTDP.

Drug investigations

Animals and drugs. Female BD IX rats (6–8 weeks-old) and female thymusaplastic *nu/nu* mice (NMRI) (20–25 g) were supplied by Zentralinstitut für Versuchstierzucht, Hannover, and maintained at 22 (S.D. 2)°C and 55 (10)% relative humidity with 12 h light and 12 h darkness in Macrolon cages with free access to autoclaved standard diet and water. NMRI *nu/nu* mice received acidified water. Cisplatin stock solution (0.5 mg/ml) was obtained from Behring Werke AG, Marburg. Buthionine sulphoximine and 3-aminobenzamide were purchased from Sigma and dissolved in 0.9% saline immediately before use. Three new metal complexes were investigated: 18-crown-6-tetracarboxybis-diammineplatinum II (CTDP) (Fig. 1), budotitane [3] and trans-imidazolium-bisimidazoletetrachlororuthenate (ICR) [2].

In vivo investigations. To compare chemotherapeutic sensitivity of O-342 and O-342/DDP, rats received intraperitoneal inoculations of about 50 mg minced tumour tissue (5×10^7 cells, day 0). On days 3, 5, 7, 10 and 13 the animals received single doses of 1.2 or 1.5 mg/kg cisplatin. This dose-range was chosen with regard to the single dose that is lethal in 10% of rats (3 mg/kg) [11]. The number of animals (not randomised) in the treated groups was 6 and in control groups 8. Combination treatment in O-342/DDP-bearing rats: after inoculation of about 10^8 tumour cells (day 0), cisplatin (single dose 1.5 mg/kg) was given once every other day for three cycles starting on day 3; two doses of buthionine sulphoximine 5 mmol/kg were given 1 day and 2 h before each cisplatin dose. The number of animals in groups cisplatin alone, cisplatin plus buthionine sulphoximine and untreated controls was 10, 8 and 6, respectively. In O-342-bearing rats, a modified schedule was used: after inoculation of 10^7 tumour cells, cisplatin (single dose 1.2 mg/kg) was started on day 3 and was given once every other day for five cycles; each cisplatin dose was preceded by one dose of buthionine sulphoximine 5 mmol/kg 2 h earlier (10 animals per group). A single dose of buthionine sulphoximine decreases the total tissue level of GSH in the liver and kidney of rats and mice to 15%–20% of untreated controls within 30–60 min [12]. To reduce the amount of drugs required, combination treatment with 3-amino-

benzamide plus cisplatin was done in NMRI *nu/nu* mice. Earlier investigations had shown that both lines grow in thymusaplastic nude mice. The two compounds were given simultaneously. Treatment started 24 h (O-342/DDP) or 48 h (O-342) after intraperitoneal transplantation of about 10^6 tumour cells. In O-342/DDP, treatment was given every other day for six courses: 3-aminobenzamide 5 mmol/kg and cisplatin 1 mg/kg per dose. The number of animals per group ranged between 9 and 12. In O-342, treatment was given every other day for three courses at the same doses. The number of animals per group ranged between 10 and 12. Animals used for combination treatment experiments were randomised.

In vitro investigations. A modification of the bilayer soft-agar assay introduced by Hamburger and Salmon was used. For cisplatin and the new metal complex compounds final concentrations of 10, 1 and 0.1 μmol/l were prepared. The test compounds were given into the top (cellular) layer at plating [13] to allow continuous exposure. For each experiment three drug concentrations and a positive reference (mercuric chloride, 100 μmol/l) were tested in triplicate (5×10^5 cells per plate). Six vehicle-treated cultures were plated for determination of control growth. Cultures were incubated at 37°C in a humidified 7.5% CO_2 atmosphere. Control plates were monitored for growth every other day with a stereomicroscope. At the time of maximum colony formation (days 7–14) final colony counts were done in an automatic image analyser (40–10-image-analysis-system, AI-Tektron-Meßsysteme, Meerbusch). 1 day before evaluation, colonies were stained with tetrazolium chloride (1 mg/ml) [14]. Objects with a circular profile and a minimum diameter of 60 μm were scored as colonies. A compound was considered active if colony formation was reduced to 30% or less of the respective control value. The resistance factor (RF) was defined as the ratio of the ID_{70} of cisplatin in O-342/DDP and O-342.

RESULTS

Tumour

Histology of the primary tumour corresponded to a granulosa cell tumour (diagnosis Prof. D. Komitowski, Institute of Experimental Pathology, German Cancer Research Center, Heidelberg). Tumour growth was highly malignant: intraperitoneal inoculation of about 50 mg tumour tissue resulted in a median survival time of about 15 days (range 14–18). The macroscopical picture was characterised by massive infiltration of organ systems within the peritoneal cavity. Histology revealed infiltration of organ structures (e.g. ovary, intestinal wall, pancreas). In the final stage, a haemorrhagic ascites developed.

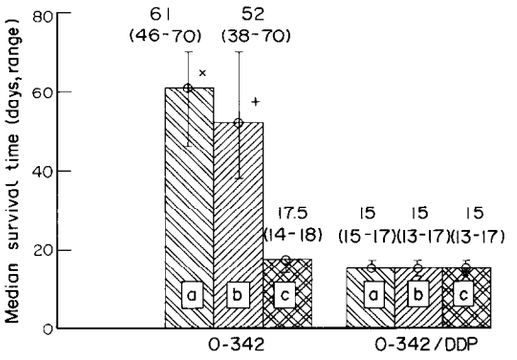

Fig. 3. Response of O-342 and O-342/DDP to treatment with cisplatin. (a) = 1.2 mg; (b) = 1.5 mg DDP/kg both for 5 doses; (c) untreated controls. x = 3/6 and + = 2/6 long-term survivors at day 70.

The level of intracellular GSH in O-342/DDP cells was approximately double that of the parental line O-342 (mean [S.E.] 1.5 [0.26] vs. 0.8[0.2] nmol/10^6 cells; $P < 0.01$, t test). Activities of GSH-related enzymes are shown in Fig. 2. There was a significant difference between the tumour lines for GSH-reductase ($P < 0.01$, t test), but GSH-S-transferase activity was similar.

Drug sensitivity

Figure 3 shows the response of both tumour lines to cisplatin *in vivo:* In animals bearing O-342, the optimal schedule (1.2 mg/kg, five doses) resulted in more than 249% increase in life span with 50% long-term survivors, underlining the high sensitivity to cisplatin. Identical treatment of O-342/DDP gave no increase in survival.

Figure 4 shows the rate of DNA interstrand cross-links in both lines induced by cisplatin at different times after exposure to 20 μmol/l for 1 h. Maximum cross-links were formed 24 h after treatment in both lines; the differences between O-342 and O-342/DDP were significant ($P < 0.05$). Cross-link formation then decreased in both lines.

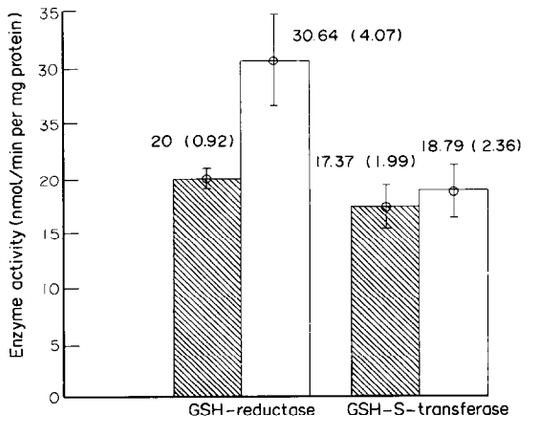

Fig. 2. Activity of GSH-reductase and GSH-S-transferase in O-342 (hatched column) and O-342/DDP (open column) cells (mean, S.E.).

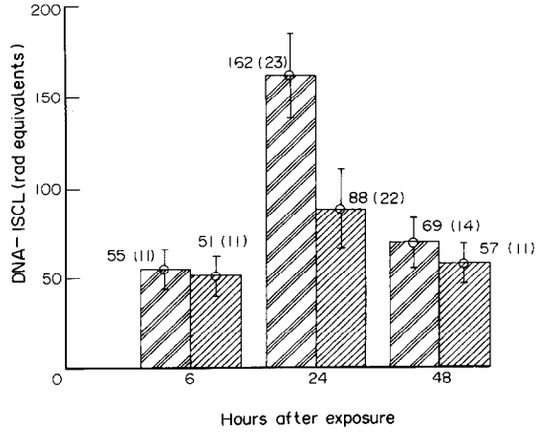

Fig. 4. DNA interstrand cross-links (ISCL) after exposure to cisplatin. Hatched column (right) = O-342 and striped column (left) = O-342/DDP.

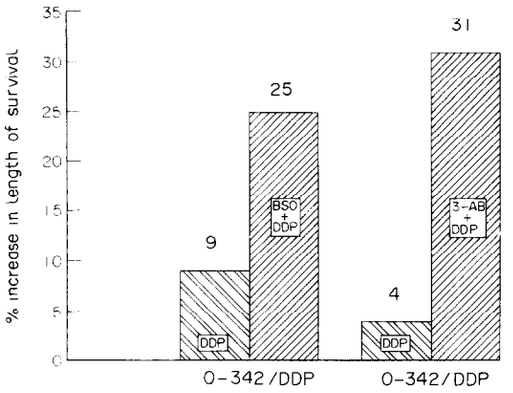

Fig. 5. Reversal of resistance of O-342/DDP to cisplatin with buthionine sulphoximine (BSO)-pretreatment (a) or by (b) 3-aminobenzamide (3-AB).

Sensitisation of tumour lines

Investigation of the combination effect of buthionine sulphoximine plus cisplatin *in vivo* was preceeded by *in vitro* studies on GSH-depletion by buthionine sulphoximine: in both cell lines a linear decrease of GSH was observed. Exposure of O-342 and O-342/DDP cells to buthionine sulphoximine 50 μmol/l over 24 h resulted in a reduction of the intracellular GSH level to 32.4 and 18.7% of untreated control values, respectively. In buthionine sulphoximine-free medium, restoration of GSH levels up to about 90% of untreated controls occurred within 24 h. (G.C. and W.J.Z.)

Results of *in vivo* combination treatment of O-342/DDP and O-342 with buthionine sulphoximine plus cisplatin are shown in Figs 5 and 6, respectively. Animals bearing O-342/DDP or O-342 showed an increase in survival with this combination. In O-342/DDP bearing animals the combination resulted in 25% vs. 9% (cisplatin alone) increase in life span ($P < 0.005$; t test, multiple analysis). In O-342 bearing animals the combination was likewise superior (320% vs. 212% increase in life span, respectively ($P < 0.05$; t test, pairwise analysis)). However, a direct comparison between response of both tumours was not possible due to different treatment schedules.

In addition, Figs 5 and 6 show results of the second approach to sensitise tumour cells with 3-aminobenzamide. In O-342/DDP, combination treatment with this drug plus cisplatin was also superior to cisplatin alone (31% vs. 4% increase in life span ($P < 0.001$; t test, multiple analysis)). Despite a lower total dosage of both drugs than in the treatment of O-342/DDP, the superiority of this combination over cisplatin alone was also observed in the sensitive line O-342 (292% vs. 225%, respectively ($P < 0.05$; t test, pairwise analysis)).

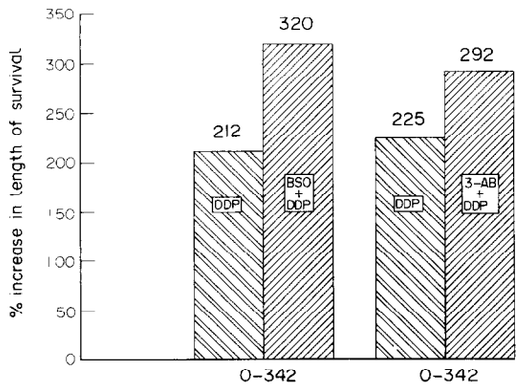

Fig. 6. Sensitisation of O-342 to cisplatin by pretreatment with buthionine sulphoximine (a) or by (b) 3-aminobenzamide (3-AB).

New metal complexes

Treatment with cisplatin caused a dose-dependent decrease of colony formation in both lines (Table 1); ID_{70} values for cisplatin were 0.31 μmol/l for O-342 and 8.7 μmol/l for O-342/DDP, thus giving an RF value of 28. CTDP had similar activity to cisplatin. In O-342/DDP, cisplatin and CTDP inhibited colony growth by more than 70% only at 10 μmol/l. These *in vitro* results for CTDP suggest a favourable therapeutic index since its LD_{50} in mice is 100 mg/kg (123 μmol/kg), whereas the LD_{50} of cisplatin is 13 mg/kg (43 μmol/kg) [15]. Budotitane showed low activity in both tumours; a dose-dependent inhibition of colony growth was not discernible. ICR showed higher activity in O-342/DDP, reducing colony formation at 10 μmol/l to 18% of controls.

DISCUSSION

GSH constitutes the majority of intracellular free thiols acting—among other important biological reactions—as protector of cells against noxious agents [16]. The acquired resistance of tumour cells to cytostatic treatment is associated with an elevation of intracellular GSH [17–20]. GSH depletion, on the other hand, either by nutritional deprivation of L-cysteine or by buthionine sulphoximine, a potent inhibitor of γ-glutamyl cysteine synthetase [21], can sensitise resistant tumour cells to different drugs [17, 18, 22–24]. Thus, with regard to cancer treatment, GSH level is an important factor for detoxification and, *vice versa*, for potentiation of anticancer drug activity.

We found that GSH levels in O-342/DDP cells were approximately double those in O-342 cells. The activity of GSH reductase was closely correlated with GSH level, which also corresponds to other observations; for example, an increase in GSH reductase activity was observed in tumour cells resistant to cisplatin, daunomycin or etoposide [25, 26]. An increase in GSH-S-transferase activity, as reported for other cisplatin-resistant tumour cells [27] was not found in our experiments.

DNA interstrand cross-links play a crucial role in cisplatin's anticancer activity [28, 29], since in most investigations the extent of cross-link formation is correlated with chemotherapeutic sensitivity. However, since this lesion accounts for only about 1% or less of the total cisplatin bound to DNA, a contribution of DNA-protein and DNA short-distance intrastrand cross-links for anticancer activity cannot be excluded [29]. Our results are in line with these data and support the importance of interstrand cross-link formation and/or persistence in cisplatin's antitumour activity since about twice as many cross-links were observed 24 h after cisplatin exposure in the highly sensitive O-342 cells than in O-342/DDP cells.

The sensitivity of both ovarian tumour lines was inversely related to their GSH levels and it was tempting to speculate that intracellular non-protein thiols play the essential role for resistance of O-342/DDP; possible mechanisms by which GSH can decrease cisplatin cytotoxicity were discussed by De Graeff *et al.* [30]. However, the approximately 2-fold higher glutathione level of the resistant line and its 28-fold higher resistance to cisplatin compared with the sensitive line suggest that resistance might only partly be due to the increased GSH level. This was supported by the observation that following a combination

Table 1. In vitro *evaluation of new metal complexes with clonogenic assay*

	Percentage control plating efficiency (mean and S.E.) O-342			0-342/DDP		
Compound	10*	1	0.1	10	1	0.1
Cisplatin	1 (1)	16 (9)	43 (7)	26 (9)	86 (12)	106 (13)
CTDP	1 (1)	12 (8)	40 (21)	18 (8)	92 (12)	106 (10)
Budotitane	58 (10)	122 (23)	79 (12)	95 (21)	100 (15)	91 (14)
ICR	48 (9)	60 (5)	91 (5)	18 (4)	65 (9)	101 (16)

*μmol/l.

treatment of O-342/DDP with buthionine sulphoximine plus cisplatin, only a limited (even though significant) increase in survival was observed compared with cisplatin alone. On the other hand, determination of the rate of DNA interstrand cross-links in O-342/DDP cells after cisplatin treatment (results not presented) with and without prior exposure of cells to buthionine sulphoximine indicated that a 70–80% depletion of intracellular GSH in the resistant line had no significant effect on DNA cross-link formation following cisplatin treatment. Hence it follows that further resistance mechanisms, which are apparently independent of intracellular GSH levels, are involved in the resistance of this tumour subline. Other causes for resistance of a tumour to cisplatin may be decreased uptake of cisplatin, increased metallothioneine levels or increased DNA repair [30–32].

The results of the combination treatment of O-342/DDP with 3-aminobenzamide plus cisplatin showed that 3-aminobenzamide, an inhibitor of poly(ADP-R)polymerase [33], significantly reversed resistance to cisplatin *in vivo*. This shows that, also in this subline, increased DNA repair apparently plays an additional role for resistance [34].

Interestingly, both sensitising drugs impressively potentiated cisplatin's antitumour activity in the sensitive line [35]. GSH deprivation can sensitize chemosensitive tumour cells *in vitro*. Suzukake *et al.* [18], for instance, showed that GSH depletion by nutritional deprivation of L-cysteine in L1210 cells increased L-phenylalanine mustard (L-PAM) sensitivity both of the L-PAM resistant and sensitive line. The same group observed *in vitro* a sensitisation of sensitive L1210 cells to L-PAM following GSH depletion by buthionine sulphoximine [22]. Hamilton *et al.* [17] reported that in drug sensitive and resistant ovarian cancer cell lines *in vitro* sensitisation to doxorubicin, L-PAM and cisplatin by buthionine sulphoximine mediated GSH depletion was higher in a drug sensitive parent line with a comparatively low baseline GSH level than in three resistant lines with increased GSH levels. Our results show that buthionine sulphoximine mediated GSH depletion can sensitise a chemosensitive experimental ovarian tumour to cisplatin *in vivo*. Altogether, our observations suggest that both sensitising measures—GSH depletion or inhibition of poly(ADP-R) polymerase—might be advantageous for the primary treatment of drug-sensitive ovarian cancer.

The activity of the new platinum compound CTDP was, on an equimolar basis, similar to or even marginally superior to cisplatin although its LD_{50} in mice is threefold higher (123 vs. 42 μmol/kg for CTDP and cisplatin, respectively), suggesting a more favourable therapeutic index for CTDP. The second result worth emphasising is the significant activity of the ruthenium compound ICR in O-342/DDP; here, ICR was superior to cisplatin. This result is in line with those reported by Garzon *et al.* [4] in experimental autochthonous colorectal tumours, which were unresponsive to cisplatin but showed significant response to ICR. The titanium complex, budotitane, which was superior to 5-fluorouracil in experimental autochthonous colorectal cancer [3] and which entered phase I trial in 1986, was inactive in both lines at the concentrations used (up to 10 μmol/l). This might be explained by its high susceptibility to hydrolysis, since our further investigations have shown that budotitane was active in O-342/DDP cells after short-term exposure to a higher concentration (100 μmol/l).

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