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Influence of Indomethacin and Difluoromethylornithine on Human Tumour Growth in Nude Mice

S. Siemer, S. Kriener, J. König, K. Remberger and O.-G. Issinger

Biopsy material from six human colorectal carcinomas was transplanted to 114 nude mice. A treatment protocol was established which included no treatment (control, C), indomethacin (I), difluoromethylornithine (D) or a combination of both (ID). The influence of the various drugs on tumour weight and protein kinase CK2 activity was monitored. CK2 activity was measured because in all tumours examined so far the enzyme activity was found to be enhanced several-fold when compared to the non-neoplastic tissue of the same patient. More than half of the investigated tumours showed a conspicuous reduction in weight after drug treatment, and I and the combination of D/I were significantly effective using the mixed model analysis. Furthermore, we have tried to discover whether there is a change in the subcellular localisation of protein kinase CK2 subunits associated with drug treatment. We analysed the tumours and the non-neoplastic control tissues by immunohistochemistry using antibodies directed against the CK2 subunits and against the proliferation marker Mib. In addition, we have also investigated the behaviour of the nucleolar protein B23 which has also been shown to be enhanced several-fold in rapidly proliferating tissue and which is also a substrate for CK2. The immunohistochemical data suggest that, irrespective of the drug treatment and the observed reduction in CK2 activity, the CK2 subunits remain localised in the nucleus.

Key words: difluoromethylornithine, indomethacin, human colorectal carcinoma, nude mice, CK2
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INTRODUCTION

AT PRESENT, the treatment of cancer results in cure in 40 per cent of patients [1]. Drug formulation and delivery play an important part in anticancer chemotherapy, by providing appropriate concentrations at sites of activity and also ensuring that local and systemic side-effects are minimised. Monotherapy is rare in cancer treatment protocols and hence there is a considerable possibility of complex interactions, both between cytotoxic drugs or between them and other drugs administered concomitantly.

We have used an approach which is based on the direct measurement of proliferation activity as measured by protein kinase CK2 activity. Protein kinase CK2 has been shown to be a proliferation-associated protein kinase (for review, see [2–4]). Furthermore, it is a ubiquitous and well-conserved protein which is strictly obligatory for cell viability [5, 6]. Hence, its inhibition will be accompanied by an overall reduction in general cellular growth. CK2 has been shown to be stimulated by polyamines like spermine and spermidine [7]. Our approach was, therefore, focused initially on the application of difluoromethylornithine, a specific inhibitor of ornithine decarboxylase (ODCase) and polyamine synthesis [8, 9]. It has been shown previously that indomethacin (I) can be used successfully in cancer therapy [10–12], especially by potentiating the effects of classical anticancer agents. The antitumour activity of I was not significantly influenced by the immunocompetence of the host. Therefore, positive effects of I treatment on tumour growth in nude mice cannot be accounted for by the immune deficiency of the animals. Since I inhibited tumour neoangiogenesis, it was postulated that this is a major mechanism for its antitumour activity [10]. I prolonged survival from 14 to 22 days in tumour-bearing mice when administered either in the drinking water or as subcutaneous (s.c.) injection [11]. This effect, owing to tumour growth inhibition, was equally effective irrespective of whether I was administered on day 1, 5, 7 or 9 following tumour implantation. I did not inhibit tumour cell growth *in vitro* [11]. The effect can only be seen in whole organisms. Specifically, I increased fatty acid synthesis when added in combination with methotrexate (MTX) [12]. Here again, the supporting effect of I could be demonstrated [12]. Inclusion of I therapy in conjunction with cisplatinum significantly enhanced the effectiveness of cisplatinum for inhibiting the growth of adenocarcinomas [13].

B23 was monitored since it is a substrate for CK2 and like CK2 is also localised in the nucleolus. If subcellular changes were to occur owing to drug treatment, the enzyme and its substrate should eventually both be affected.

Colorectal tumours with an incidence of 25/100 000 in the western population are the second most frequent tumours of all carcinomas. Surgery is the only method of cure. Results of adjuvant chemotherapy and pre- or postoperative radiation are disappointing.

In the present study, we attempted to establish a potent chemotherapy for colorectal carcinomas with lower side-effects. Different colorectal carcinomas were transplanted into 114 nude mice. In addition to single drug treatment with I and D, we also used a combination therapy of both drugs. Tumour weight alone may not always reflect the obtained therapeutic effects, since it may well be that tumour size remains almost unaltered yet the cells become necrotic and lose metabolic activity. The rationale behind the combined application of I and D is partly owing to the fact that CK2 is very much stimulated by polyamines, e.g. spermine and spermidine, and that the inhibition of ODCase by D, potentiated by I, might be a promising chemotherapeutic approach. Therefore, we included the protein kinase CK2 activity determination which should be helpful in obtaining additional information on the metabolic state of the tumour.

The comparatively small side-effects in the animal experiments as compared to other adjuvant concepts may be indicative of an additional increase in life quality.

MATERIALS AND METHODS

Antibodies and staining conditions

The antibodies used throughout the experiments were B-4, polyclonal antibody raised in rabbit and directed against the recombinant human CK2 β-subunit (dilution 1:5), and B23, murine monoclonal antibody (25-fold dilution). B23 antibody was kindly provided by Dr Chan (Baylor College, Houston, Texas, U.S.A.). Both antibodies were used on cryostat sections which were formaldehyde fixed for 5 min followed by H_2O_2 (0.3%) treatment for 15 min. The Mib antibody was from Dianova (Hamburg, Germany). Mib is a murine monoclonal antibody used for the detection of Ki67 antigen in paraffin sections. Paraffin sections underwent deparaffination, rehydration and microwave treatment for 5 min twice (dilution 1:30). The secondary antibody used for the detection of B-4 was a biotinylated swine antirabbit antibody. B23 and Mib were detected using a biotinylated rabbit antimouse secondary antibody. All secondary antibodies were from Dako (Denmark).

Heterotransplantation of tumours on nude mice

Most human tumours were obtained as fresh surgical specimens. The tumours were cut into small, 1-mm^3 fragments and implanted s.c. into nude mice. To conserve mice, each mouse was injected at two sites, once on each flank [14]. No difference between the growth on one side and growth on the contralateral side was observed.

Procedures and design of the trial

Tissue from six human colorectal carcinomas was transplanted into 114 mice as described earlier. When transplantation proved successful, a treatment protocol was established which included mice which received no treatment (C, control), I, D or a combination of both (ID). Because of limited resources, the four treatments were not administered for all human tumours. All six tumours were tested under C treatment, one tumour was also tested under D and ID treatment, another one was tested with D and C only and the remaining four were tested with I and C. Sample sizes within the treatment tumour group range from five to 17.

Statistical methods

The trial was conducted as a randomised incomplete block design with repeated measurements within each block. Some, but not all, treatments were applied to several mice with transplants from the same patient [15, 16].

With such a design, it is possible to decide whether a treatment effect is constant over different blocks. The appropriate F-test

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in a linear model is referred to as interaction test in the sequel. If there is a differential treatment effect depending on the human tumour, an adequate statistical test of a main treatment effect has to account for the variation of treatment differences between the tumours. This is accomplished by specifying a mixed linear model with random tumour effect and a random treatment-by-tumour interaction [15, 16]. The statistical test results are usually less significant than in a fixed effect model, but allow inferences to be made from the six tumours studied here to other human colorectal carcinomas.

Analysis starts with description of treatment effects within single tumours. Data are presented in the form of box plots [17]. The box depicts the first and third quartile; outliers more than 1.5 interquartile ranges far from the respective quartile are marked separately. Lines are drawn from the extreme non-outlying observations to the box. The median and mean are marked by a vertical bar and a cross, respectively. For treatment comparisons within single tumours, unpaired *t*-tests are performed.

Analysis of overall treatment effects is accomplished by mixed linear model analysis [15, 16] as follows.

As a consequence of the peculiar pattern of treatment allocation, pairwise overall comparisons were not performed in a uniform fashion. The comparison between I and C was carried out on four tumours tested under both I and C treatment. The comparison between D and C was performed on two tumours tested on both treatments. Out of the three treatments the comparison of the ID treatment with D and C was done on only one tumour.

The comparison between D and I was carried out by comparing D/C differences in two tumours with I/C differences in the remaining four tumours (by definition of the appropriate contrast in a mixed model).

Owing to the study design, the assessment of the effect of ID treatment cannot account for variation between tumours since it was tested on one tumour only. Thus, conclusions as to how the ID treatment will perform on other tumours are limited.

The logarithm of the response variable tumour weight was taken to the base 10 in order to improve homogeneity of variances. Because there were only five tumours with weight measurements (for tumour no. 2 the weight was not determined), all five groups of mice (numbers 1, 3–6), receiving I or ID treatment were compared to five control groups within tumours of the same origin by means of a mixed model analysis. Results were retransformed into weights and, consequently, retransformed differences are reported as weight reduction factors.

Proteins and antibodies

CK2-Protein kinase CK2 is a tetramer ($\alpha_2\beta_2$). The α-subunit is the catalytic subunit, the β-subunit confers stability, activity and specificity [4]. The antibodies used in this study were derived from rabbits and are directed against the recombinant human β-subunit. CK2 has been shown to be constitutively expressed in all cells so far examined. However, in proliferating cells its activity is elevated 3–5-fold.

The nucleolar protein B23 has been given a number of different names: B23, NO38, numatrin [18]. It is a substrate for CK2 located predominantly in the granular component of the nucleolus and, therefore, likely to function at later stages of ribosome biogenesis. It is speculated that it may play a role in the assembly of preribosomal particles and thus be functionally related to the nucleosome assembly factor, nucleoplasmin [19]. The amount of B23 increases 5–20-fold in proliferating cells [20]. The monoclonal antibody directed against B23 was a gift from Dr P.K. Chan [21].

The antibody Mib is directed against a so far unknown nuclear antigen which has been shown to be predominantly present in mitotically active cells, hence it was established as a proliferation marker. The antibody used in this study is from Dianova (Hamburg).

Immunostaining

For immunostaining, the samples, which had been kept frozen in liquid nitrogen, were sectioned at 5 μm at −20°C, fixed without drying for 5 min in buffered formalin (pH 7.4) and finally rinsed in phosphate-buffered saline, pH 7.2. The endogenous peroxidase was blocked with 0.3% H_2O_2 prior to application of the first antibody. The slides were incubated with the primary antibody for 1 h at room temperature. The second antibody and an avidin–biotin complex (ABComplex/HRP, Dako) were applied next and incubated for 30 min at room temperature. The reaction was visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB), leaving an intensely brown-coloured end product.

Staining procedure of paraffin-embedded tissue

After deparaffination and rehydration in gradate alcohols, the slides were transferred to 10 mM citrate buffer, pH 6.0, in a plastic jar and then microwaved twice for 5 min. After the last treatment, the tissue sections were left in the buffer for 15–20 min at room temperature.

Negative controls were performed by replacing the primary antibodies with a non-immune serum of the mouse (Mib, B23) or with preimmune serum (B-4, anti CK2β-antibody).

RESULTS AND DISCUSSION

Tumour weight

The weight distribution of the tumours from the different groups is displayed by boxplots (Figure 1). Computation of means (marked by X) is performed on log weight values. There is a marked dependency on tumour origin and, consequently,

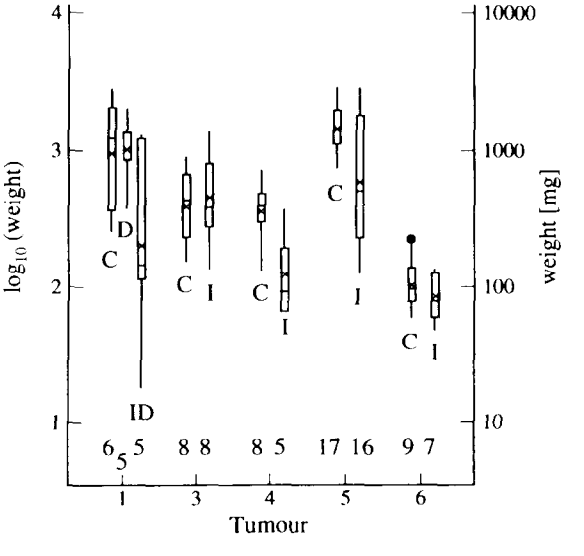

Figure 1. Tumour weight by implanted tumour and treatment. C, control; D, difluoromethylornithine; I, indomethacin; ID, combination. Numbers near the horizontal axis indicate sample sizes, means (of log weight) are marked by X. No measurements on tumour no. 2.

the typical patient heterogeneity with respect to weight. Hence, comparison between the control groups is highly significant ($P < 0.0001$).

The effect of active treatment as compared to the control is well expressed in tumour no. 1 (ID/C), $P = 0.11$, an average reduction by factor 0.23 and tumours no. 4 and no. 5 (I/C), $P < 0.01$ and $P < 0.003$, average reduction by factor 0.34 and 0.41, respectively. There appears to be no effect of D on tumour no. 1 and of I in tumour no. 3 and no. 6 (Figure 2).

The combination treatment and D can be compared only in tumour no. 1 where the reduction factor (ID/D = 0.21; $P = 0.10$) has the same order of magnitude as the reduction by combination treatment compared to the control.

When analysing the ID treatment and I (taken as one kind of treatment) in comparison with C in a mixed model (with random treatment-by-tumour interaction), heterogeneity of treatment effects cannot be precluded ($P = 0.07$) and the average reduction factor is 0.49, being significantly different from 1 ($P = 0.05$). On the whole, the combination treatment showed the largest weight reduction (Figure 2).

CK2 activity

Figure 3 shows the distribution of CK 2 activity in all groups of mice according to the treatment protocol and transplanted human tumours. Pairwise comparisons within the tumours are presented in Figure 4, where mean differences and 95% confidence intervals are displayed (confidence intervals that do not intersect the zero-reference line indicate significant differences at 5% level).

As in the case of tumour weight, there is a significant difference between the control group ($P < 0.0001$ for comparison of six control groups), the mean CK2 activity ranging between 655 for tumour no. 4 and 1735 for tumour no. 1.

All mean differences in CK2 activity between treatment and control as well as the difference between ID treatment and D are negative (see Figure 4). The differences between I treatment and the controls range between −476 and −44.1, average to −250, and are significant ($P < 0.001$) for two out of four tumours. The differences between D and C are −243 and −44.1 ($P = 0.052$ and $P = 0.56$) and average to −144. ID treatment and C differ by −302 ($P < 0.01$). The small observed difference of −58.8 between ID and single D treatment is not significant.

Mixed model analysis of variance shows that the heterogeneity of treatment effects is significant among the four tumours numbers 3–6 where I is compared to C ($P < 0.01$). Thus, I performs differently on different human tumours, yet there are tumours where CK2 is affected.

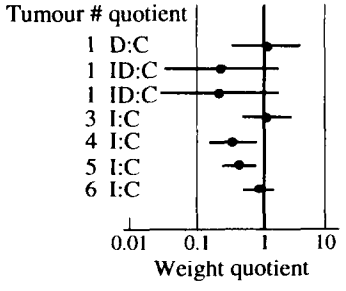

Figure 2. Within-tumour comparisons of treatments, pairwise quotients and 95% confidence intervals based on mean differences of log weight.

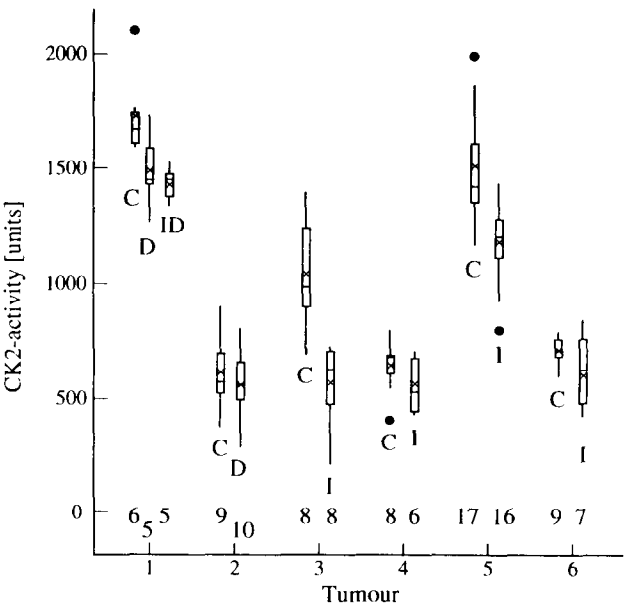

Figure 3. CK2 activity by implanted tumour and treatment. C, control; D, difluoromethylornithine; I, indomethacin; ID, combination. Numbers near the horizontal axis indicate sample sizes, means are marked by X.

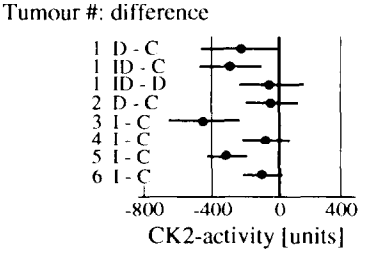

Figure 4. CK2 activity within tumour comparisons of treatments. Pairwise differences and 95% confidence intervals.

A similar heterogeneity can be observed for the comparison of D and C in tumours 1 and 2 but this is not significant ($P = 0.13$).

The average treatment/control differences are −250 for I (in tumours 3–6; $P = 0.04$) and −143.7 for D (in tumour no. 1 and no. 2; $P = 0.39$).

A comparison between D and I is made indirectly by comparing the average I/C difference of −250 (seen in tumour numbers 3–6) with the D/C difference of −144 (seen in tumour no. 1 and no. 2). However, the differences are not significant ($P = 0.53$).

The difference ID/C of −302 in tumour no. 1 is higher than the average difference I/C and is less clear than the sum of the average differences I/C and D/C. It cannot be proven statistically whether ID therapy is more effective than single therapy, nor whether the effect of combination treatment is additive, subadditive or supra-additive (synergistic). Clearly, it is necessary to pursue these promising experiments using the combination treatment with I in order to enhance susceptibility of tumour cell response towards chemo- and radiation therapy. In particular, the role of prostaglandin synthesis inhibition in this context should be explored in order to better understand the basis for this supporting effect of I and leading to a direct comparison of the combination therapy with single treatments within several human tumours.

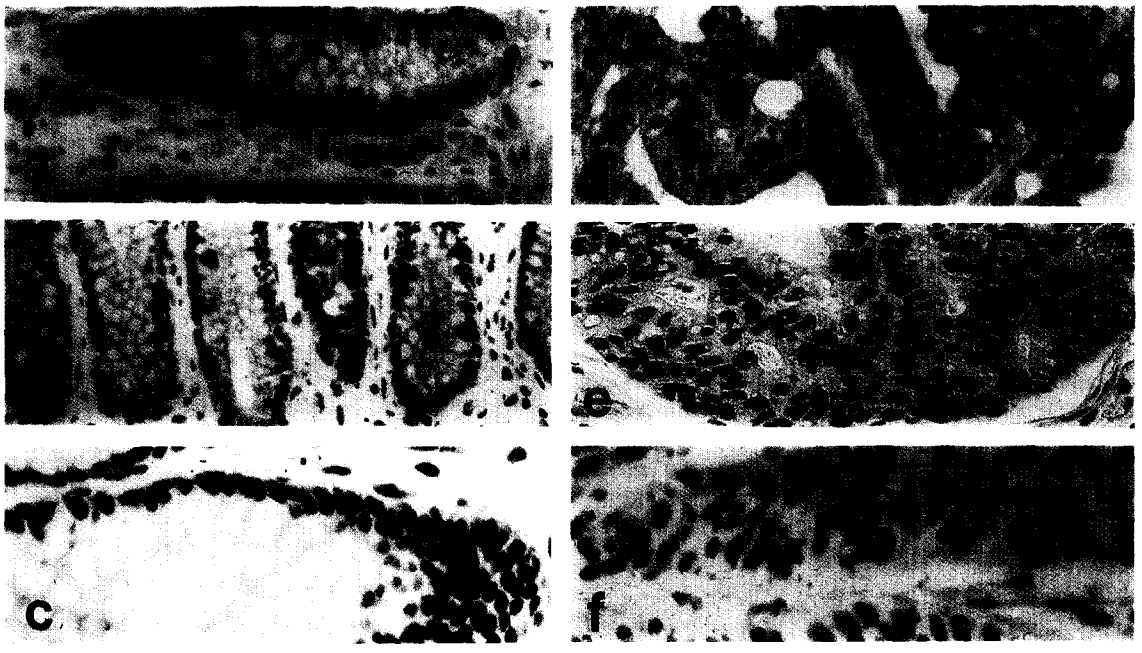

Figure 5. (a–c) Human colonic mucosal epithelium presenting: (a) a positive nuclear staining of all cells with B-4, magnification 250-fold; (b) an irregular Mib-positive nuclear staining in the proliferating zone, magnification 250-fold; (c) several B23-positive nucleolar spots in the nuclei of all crypt cells, magnification 500-fold. (d–f) Heterotransplanted colorectal carcinoma (GII) showing: (d) a different intensity of positive nuclear staining of all cells with B-4, magnification 250-fold; (e) an intense nuclear staining of Mib-positive cells, magnification 250-fold; and (f) B23-positive nucleolar spots of all tumour cells, magnification 500-fold.

Immunohistochemical analysis

Tissue sections from the heterotransplanted human tumours were prepared and stained for the presence of CK2 subunits α and β. In addition, we also looked for the proliferation marker Ki67/Mib. The tested antigens were localised in the nucleus. Treatment with the different drugs did not lead to a significant change in nuclear localisation as it is the case in tissue cultures when they are in Go phase.

Figure 5(a–c) shows human colonic crypt cells stained with three different antibodies. Figure 5a shows staining with anti-CK2β antibody, B-4. It can be seen that this antibody leads to an exclusive nuclear staining of the mucosal epithelium including goblet cells, irrespective of the proliferationally active zone, i.e. the lower one third of the crypt. For control purposes, staining was carried out with Mib antibody, an established proliferation marker (Figure 5b). We can see clearly that some of the lower crypt cells become stained. Figure 5c shows staining of the crypt cells with an anti-B23 antibody, giving rise to the typical nucleolar spots and patches as described elsewhere [18]. The overall staining, similar to that of the CK2β antibody, is not so surprising since B23 has been shown previously to be an excellent substrate for CK2 and a co-localisation must occur at some time. In Figure 5 (d–f) the three antibodies were used to stain a colorectal carcinoma which had been heterotransplanted into nude mice. Again, the antibodies directed against CK2β (Figure 5d) and B23 (Figure 5f) stain virtually all cells, as was the case in the non-neoplastic tissue. In contrast to the staining in the normal colon, the Mib antibody stains virtually all cells in the colorectal carcinoma. This was expected because colorectal carcinomas are very actively proliferating tumours (Figure 5e). Clearly, all three antibodies behave differently: whereas anti-B23 antibody is detecting its antigen in all tissues and the carcinoma investigated (Figure 5c, f), the CK2 antibodies behave in part like the classical proliferation marker Mib, although other cells are detected beside proliferating ones, indicating additional characteristics for the CK2 antigen.

In the present work, an effective chemotherapy concept for colorectal carcinomas is presented. The combination therapy using I and D shows the most significant weight reduction of the tumours and a significant reduction of CK2 activity. The treated tumours are not only smaller than those of the control group, eventually owing to reduced necrotic material, but also exhibit a reduced tendency for proliferation. The above-applied statistical approach was carefully chosen in order to ensure an adequate statistical analysis of the parallel tumour model with hetero-transplantation on the nude mouse [15, 16]. Many analyses apply *t*-test to pooled data or similar statistical methods which would yield invalid conclusions. The combination therapy did not lead to any observable side-effects in animals which will also increase life quality in man once clinically tested.

Further studies will be conducted in order to further assess validation of the combination therapy with the final goal of a clinical application.

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Relationships Between Resistance to Cisplatin and Antifolates in Sensitive and Resistant Tumour Cell Lines

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Possible relationships between tumour resistance to cisplatin and the folate-based thymidylate synthase (TS) inhibitors, CB3717 and ZD1694 (tomudex), have been investigated *in vitro* using a panel of tumour cell lines (predominantly human ovarian), either parental or possessing acquired resistance to cisplatin or ZD1694. Across eight parent human tumour cell lines, ZD1694 was the most potent drug (mean IC_{50} of 1.9×10^{-8} M), being over 250 times as potent as its prototype CB3717 (mean IC_{50} of 4.8×10^{-6} M). In five pairs of acquired cisplatin-resistant human tumour cell lines (three ovarian, one cervical and one testicular) which encompass all of the main known mechanisms of platinum drug resistance, ZD1694, CB3717 and the DHFR inhibitor, methotrexate, all exhibited non-cross-resistance. The cervical line, HX/155cisR, showed collateral sensitivity to ZD1694, CB3717, 5-fluorouracil (FUra) and fluorodeoxyuridine (FdUrd). One cell line, A2780cisR, showed a low level of cross-resistance to FUra (resistance factor, RF, of 1.5) and FdUrd (RF of 3.8). A2780cisR, in common with two other cisplatin-resistant lines, did not possess elevated TS activity compared with its parent. Cisplatin retained activity in four acquired ZD1694-resistant cell lines (encompassing reduced folate transport, elevated TS and defective polyglutamation mechanisms of resistance). Furthermore, combinations of ZD1694 with each of the platinum-based drugs, cisplatin, carboplatin and the recently introduced orally administrable, JM216, all showed additive growth inhibitory effects by median effect analysis. These data suggest that the tumour inhibitory properties of the recently introduced highly potent TS inhibitor, ZD1694, and cisplatin, and, moreover, their respective mechanisms of resistance, do not overlap. Therefore, these drugs may be considered for combination in the clinic.

Key words: tomudex, platinum, antifolates, resistance
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