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Modulation of Cisplatin Cytotoxicity by Human Recombinant Interferon-γ in Human Ovarian Cancer Cell Lines

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Cytotoxic interactions between recombinant human interferon-γ (IFNγ) and cisplatin have been studied in six ovarian cell lines (IGROV1, NIHOVCAR3, SKOV3, OVCCR1, 2008 and its cisplatin resident variant 2008/C13*). Studies were performed using a cell survival assay. Results were assessed using median effect analysis. Synergy between these two drugs was observed in cell lines sensitive to IFNγ, whatever their relative sensitivity or resistance to cisplatin, suggesting that IFNγ enhances the cytotoxic activity of cisplatin. This interaction is not due to an increase in platinum accumulation in cells. This combination of drugs should be evaluated against human ovarian cancer xenografts in nude mice before its use in clinical practice.

Key words: interferon-γ, cisplatin, interactions, median effect analysis, human ovarian cancer cell lines, combination index, synergism

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INTRODUCTION

THE INCORPORATION of biological agents, often termed biological response modifiers (BRM) into combination regimens with standard chemotherapeutic agents offers an important challenge to medical oncologists, since the assumptions for their use are likely to differ from those for chemotherapeutic agents. The interferons (IFN) are a family of naturally occurring glycoproteins which share antiviral, immunomodulatory and antiproliferative effects. Interferon-γ (IFNγ) has a somewhat more potent antitumour activity than the other classes of interferons

(α, β) [1]. This IFN affects the proliferation of ovarian carcinoma cell lines *in vitro* and the growth of human ovarian xenografts *in vivo* [2, 3]. Although only a few clinical trials have been conducted in ovarian cancer with IFNγ, they have demonstrated the efficiency of this drug [4, 5]. Activity has been shown when this BRM was administered intraperitoneally (i.p.) [6, 7]. Such a result might be explained by the fact that IFNγ might exert its immunomodulatory activity via immunocompetent cells present in the peritoneum [8].

Cisplatin is the cornerstone of the chemotherapeutic treatment of ovarian cancer. Its pharmacological advantage, shown after i.p. administration, allows the application of the concept of dose intensity [9]. Unfortunately, the clinical utility of cisplatin has been limited by the frequent development of resistance and/or limiting toxicities [10]. Research studies have been developed with the aim of potentiating cisplatin activity. Few have investigated the interactions of IFNγ and cisplatin *in vitro*: Saito and colleagues [11] demonstrated additive effects on the BG-1 human ovarian cancer cell line, but the concentrations of IFNγ were not within the range of levels that can be attained in plasma or peritoneal fluid. More recently, Marth and colleagues [12] showed a synergistic effect for A2780 and HTB-77 ovarian cell lines, and only an additive effect for the OVCAR-3 ovarian cell line.

In this study, we have evaluated the cytotoxic activity of human recombinant IFNγ in combination with cisplatin on a panel of six different human ovarian carcinoma cell lines corresponding to the main histological types of ovarian cancer: serous and endometrioid. The interactions, determined by median effect analysis, were studied on two variants of the same cell line different in their sensitivity to cisplatin.

MATERIALS AND METHODS

Cell lines and cell culture

Six human ovarian carcinoma cell lines were used in this study: SKOV3 and NIHOVCAR-3 were obtained from the American Type Culture Collection (Rockville, U.S.A.). IGROV1 was a gift from Dr J. Bénard [13]. 2008 [14] and its 10-fold cisplatin-resistant (2008/C13*) subline [15, 16] were generously provided by Dr Stephen B. Howell. OVCCR1 was established in our laboratory from the peritonal fluid of a patient suffering from ovarian cancer [17].

Cell lines were maintained in RPMI 1640 medium (Gibco, France) supplemented with 5% (v/v) fetal bovine serum (FBS) (Seromed, France), insulin (5 μg/ml) and epidermal growth factor (EGF, 2 ng/ml) (Boehringer-Mannheim, Germany), and kept at 37°C in a humidified atmosphere containing 5% CO_2.

Drugs and chemicals

Cisplatin (powder 10 mg) was obtained from Roger Bellon Laboratories (Neuilly-sur-Seine, France). The stock solution was stored at 4°C, and working solutions were prepared immediately before use by dilutions in the culture medium. IFNγ was derived from human recombinant DNA, and obtained from the E. Boehringer Institute (Vienna, Austria). The preparation was essentially pure and had a specific activity of 2×10^7 U/mg of protein. The stock solution was stored at −20°C, and the working solution was prepared before use by dilutions in the culture medium.

Cell survival assay

Cells growing in log phase were trypsinised, dispersed and plated onto plastic tissue culture dishes in triplicate at a density of 50 000 cells/dish. The cells were allowed to attach for 24 h (SKOV3, IGROV1, 2008 and 2008/C13*) or 48 h (OVCCR1 and NIHOVCAR-3), then the medium was changed to one containing the appropriate concentrations of IFNγ, cisplatin or different combinations of these substances.

For all experiments, the exposure time was 1 h for cisplatin, whereas the BRM was present in the culture medium for the whole culture period. After three doubling times, the cells were collected and numerated using an electronic particle counter (Coulter Electronics, Margency, France). The results were expressed as percentages of survival cells, and the concentrations of cisplatin or IFNγ which caused 20, 50 or 80% growth inhibition with respect to controls (IC_{20}, IC_{50} and IC_{80}) were determined.

Median effect analysis

Median effect analysis was used to determine the interactions between cisplatin and IFNγ [18]. Combination index (CbI) was determined from a cell survival assay at increasing levels of cell kill. CbI values less than or greater than 1 indicate synergy and antagonism, respectively, whereas a CbI value of 1 indicates additivity of the drugs. Cisplatin and IFNγ were combined in a fixed ratio corresponding to the ratio of the individual IC_{50} (w/w) for each cell line (IFNγ/cisplatin): 1/1600 for OVCCR1, 1/128 for NIHOVCAR-3, 1/1000 for 2008, 1/2250 for 2008/C13*, 1/200 for SKOV3. For IGROV1, the ratio was fixed to 1/800. The cytotoxicity was compared to the cytotoxicity of each drug alone in every experiment, and each experiment was performed three times using triplicate cultures for each data point.

Platinum accumulation

Cells growing in log phase in Falcon cell culture flasks (Nunclon, France) (175 cm^2) were harvested and plated onto cell culture flasks (75 cm^2) at a density of 6×10^6 cells/flask. Twenty-four or forty-eight hours later, the cells were treated with a fixed concentration of cisplatin for 1 h with or without IFNγ. The cells were washed three times with phosphate buffered saline (PBS), trypsinised, counted and centrifuged at 4°C for 15 min at 1000 rpm. The supernatant was discarded and the cellular pellet was recovered with 5 ml of PBS and recentrifuged; the supernatant was discarded and the final pellet was reconstituted with 300 μl H_2O. The cells were disrupted by sonication (6–10 s) and 60 μl of this final solution was used for platinum measurement by flameless atomic absorption spectrophotometry.

RESULTS

Cytotoxicity of cisplatin and IFNγ in vitro

The cytotoxicity was assayed against six ovarian cell lines in culture. The IC_{20}, IC_{50} and IC_{80} for cisplatin and IFNγ are summarised in Table 1. The IC_{50} of cisplatin differed from one cell line to another, ranging from 0.4 to 9 μg/ml; in particular, the IC_{50} of cell line 2008 and its cisplatin-resistant subline (2008/C13*) were 1 and 9 μg/ml, respectively.

A similar heterogeneity in cytotoxic response was noticed with IFNγ: four cell lines (OVCCR1, NIHOVCAR3, 2008 and its

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Table 1. Relative cytotoxicity of cisplatin (1-h exposure) and IFNγ (continuous exposure) on six human ovarian cancer cell lines evaluated by the cell survial assay

	Cisplatin (μg/ml)			IFNγ (U/ml)		
	IC_{20}	IC_{50}	IC_{80}	IC_{20}	IC_{50}	IC_{80}
OVCCR 1	0.35 ± 0.05	0.80 ± 0.10	1.75 ± 0.25	5 ± 3	10 ± 1.5	17.5 ± 2.5
NIHOVCAR 3	0.15 ± 0.05	0.48 ± 0.08	0.75 ± 0.09	12.5 ± 2.5	75 ± 12	225 ± 25
IGROV 1	0.07 ± 0.02	0.40 ± 0.09	1.25 ± 0.25	N.E.	N.E.	N.E.
SKOV 3	1.56 ± 0.10	3.00 ± 0.18	6.51 ± 0.50	200 ± 20	300 ± 25	N.E.
2008	0.40 ± 0.10	1.00 ± 0.18	2.00 ± 0.21	7 ± 2	20 ± 3.5	61 ± 8
2008/C13*	2.00 ± 0.15	9.00 ± 0.37	21.9 ± 0.72	10 ± 1	80 ± 17	620 ± 10

Values represent the average of three independent determinations carried out in triplicate. Means ± S.D.are given. IC_{20} = 20% inhibitory concentration. IC_{50} = 50% inhibitory concentration. IC_{80} = 80% inhibitory concentration. N.E., not evaluable.

subline C13*) might be considered sensitive (IC_{50} < 100 U/ml), two others do not respond to physiological concentrations, and for IGROV1 the IC_{50} could not be determined.

Effect of cisplatin in combination with IFN-γ

The nature of interactions between cisplatin and IFNγ was investigated using a schedule of drug exposures that mimicked the clinical conditions upon which this study was based. In the clinical situation, the protocols are started with a short infusion of cisplatin and a repeated weekly administration of IFNγ. To reflect this schedule, the ovarian cell lines were treated for 1 h with cisplatin and exposed continuously to IFNγ.

The CbI values determined by using the technique of median effect analysis for obtaining 20, 50 and 80% inhibition of growth in the different cell lines are summarised in Table 2. For OVCCR1 and NIHOVCAR3 (Figure 1), the CbI_{50} values were 0.81 ± 0.06 and 0.62 ± 0.11, respectively, indicating that, for these two cell lines, the combined amount of the two drugs necessary to kill 50% cells was only 0.81 and 0.62 times as much as would be required if they demonstrated purely additive behaviour. Figure 2 shows the average plot of CbI as a function of fraction affected derived from three experiments with IGROV1 and SKOV3, and it indicates that there was a moderate degree of antagonism, particularly at the lower level of cell kills, for IGROV1 and at higher levels for SKOV3.

The nature of interactions was also investigated using two variants of the same cell line differing in their sensitivities to cisplatin. The CbI_{80} values were 0.74 ± 0.09 for cell line 2008 and 0.36 ± 0.08 for the platinum resistant subline 2008/C13* (Figure 3)($P < 0.05$).

Cytotoxic activities of the two compounds, separately or in combination, on the different cell lines are summarised in Table 3, which suggests that synergy between cisplatin and IFNγ is independent of the cell line doubling times, but may be crucially dependent on the IFNγ sensitivity.

Platinum accumulation

Platinum uptake has been tested only in cell lines in which synergism has been noted. The same fixed ratio of concentrations of the two drugs used for median effect analysis was used. Cells

Table 2. Values of combination index for the interaction between cisplatin and IFNγ on six ovarian cancer cell lines

	CbI 20%	CbI 50%	CbI 80%
OVCCR1	0.72 ± 0.04	0.81 ± 0.06	0.93 ± 0.09
NIHOVCAR3	0.93 ± 0.14	0.62 ± 0.11	0.49 ± 0.16
IGROV1	1.97 ± 0.37	1.30 ± 0.05	1.06 ± 0.43
SKOV3	1.22 ± 0.11	1.45 ± 0.15	1.71 ± 0.19
2008	1.20 ± 0.07	0.82 ± 0.05	0.74 ± 0.09
2008/C13*	1.18 ± 0.11	0.56 ± 0.08	0.36 ± 0.08

CbI 20% = combination index indicating the combined amount of the two drugs necessary to kill 20% of cells. CbI 50% = combination index indicating the combined amount of the two drugs necessary to kill 50% of cells. CbI 80% = combination index indicating the combined amount of the two drugs necessary to kill 80% of cells. Values represent the average of three independent determinations using triplicate cultures for each data point. Means ± S.D. are given. CbI < 1 indicates synergy.

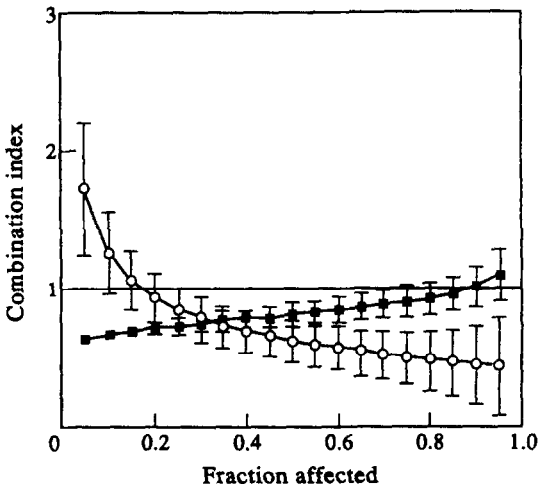

Figure 1. Combination index plot as a function of fraction affected by the interaction between cisplatin and IFNγ on OVCCR1 and NIHOVCAR3 ovarian cell lines. ■ Effect of cisplatin and IFNγ on the OVCCR1 ovarian cancer cell line at a concentration ratio of 1/1600. ○ Effect of cisplatin and IFNγ on the NIHOVCAR3 ovarian cancer cell line at a concentration ratio of 1/128. Each curve represents the average of three separate experiments using triplicate cultures for each data point. Mean ±S.D. are given. CbI < 1 indicates synergy.

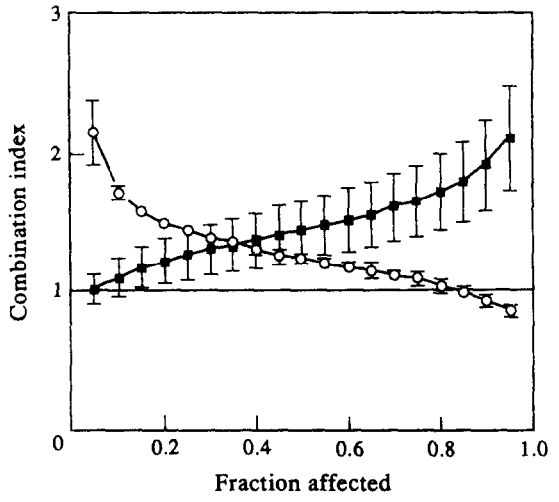

Figure 2. Combination index plot as a function of fraction affected by the interaction between cisplatin and IFNγ on SKOV3 and IGROV1 ovarian cell lines. ■ Effect of cisplatin and IFNγ on the SKOV3 ovarian cancer cell line at a concentration ratio of 1/200. ○ Effect of cisplatin and IFNγ on the IGROV1 ovarian cancer cell line at a concentration ratio of 1/800. Each curve represents the average of three separate experiments using triplicate culture for each data point. Means ±S.D. are given. CbI < 1 indicates synergy.

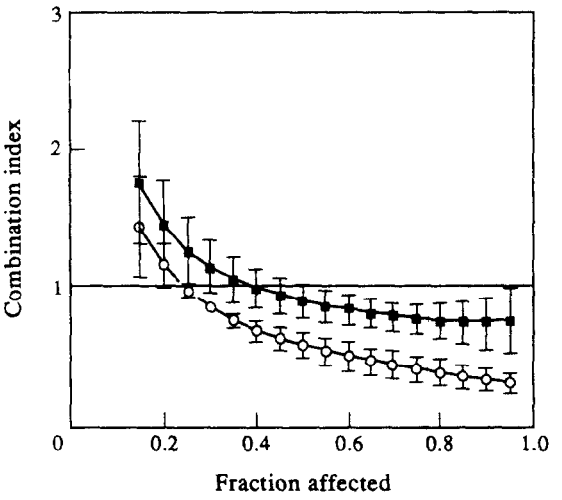

Figure 3. Combination index plot as a function of fraction affected by the interaction between cisplatin and IFNγ on 2008 and 2008/C13* ovarian cell lines. ■ Effect of cisplatin and IFNγ on the 2008 ovarian cancer cell line at a concentration ratio of 1/1000. ○ Effect of cisplatin and IFNγ on the 2008/C13* ovarian cancer cell line at a concentration ratio of 1/2250. Each curve represents the average of three separate experiments using triplicate cultures for each data point. Means ±S.D. are given. CbI < 1 indicates synergy.

were incubated with cisplatin IC_{50} in the presence or absence of the corresponding concentrations of IFNγ. Another concentration corresponding to the cisplatin IC_{80} for NIHOVCAR3, 2008 and 2008/C13* and to the cisplatin IC_{20} for OVCCR1 was also tested because of the shape of the interaction curves.

As shown in Table 4, no major difference was observed in terms of platinum uptake in any cell line, whatever the treatment and the concentration tested.

DISCUSSION

Drugs that enhance the sensitivity of tumour cells to cisplatin are of particular interest both because of the possibility of using them to potentiate the antitumoral activity of cisplatin in patients, and because they provide a pharmacological tool with which to dissect the complex mechanisms by which cells protect themselves against the cytotoxic agent.

This study demonstrates marked cytotoxic synergy between IFNγ and cisplatin in four out of six human ovarian cancer cell lines, using a formal analytical technique (median effect analysis) to elucidate the nature of interactions. The synergistic interaction was observed at all levels of effects over the first two logs of cell kills and, in particular, was observed at low concentrations of both cisplatin (1 μg/ml) and IFNγ (100 U/ml), which are well within the range that can be attained in human plasma or peritoneal fluid using standard dosing regimens [7, 9]. Interestingly, synergy between cisplatin and IFNγ was observed using the subline 2008/C13*, that was 10-fold more resistant to the cytotoxic effect of cisplatin. Measurement of *in vitro* sensitivity in ovarian carcinoma tumour samples or cell lines obtained before or after treatment of patients with cisplatin indicated that the level of resistance that emerges *in vivo* is quite modest, in the range of 1.5–3.0-fold [19, 20]. Increased drug exposure by i.p. administration of platinum derivatives or modulation of cisplatin activity, by IFNγ for instance, might overcome resistance, and produce prolonged survival in patients with some types of ovarian carcinoma [9]. In contrast, no synergism was observed using the two cell lines which are not sensitive to IFNγ: IGROV1

Table 3. Comparative data of the different ovarian cell lines concerning their biological characteristics, their relative sensitivity to cisplatin, IFNγ and the consequence on the drug combination

Cell line	Histological type	Doubling time (h)	Cisplatin sensitivity*	IFNγ sensitivity†	Synergism‡
OVCCR1	Serous	72	++	+++	+
NIHOVCAR3	Serous	48	+++	++	++
SKOV3	Endometrioid	24	+	−	−
IGROV1	Endometrioid	24	+++	−	−
2008	Serous	30	++	+++	+
2008/C13*	Serous	24	−−−	++	+++

*Cisplatin sensitivity was assessed as: − IC_{50} > 5 μg/ml; + 1 < IC_{50} < 5 μg/ml; ++ 0.5 < IC_{50} < 1 μg/ml; +++ IC_{50} < 0.5 μg/ml. †IFNγ sensitivity was assessed as: − IC_{50} > 250 U/ml; + 100 U/ml < IC_{50} < 250 U/ml; ++ 50 U/ml < IC_{50} < 100 U/ml; +++ IC_{50} < 50 U/ml. ‡Synergism was assessed as: −CbI 50% > 1; + 0.8 < CbI < 1; ++0.6 < CbI < 0.8; +++ CbI < 0.6.

Table 4. Platinum accumulation in four ovarian cancer cell lines treated with the cisplatin with or without cotreatment with IFNγ. Data only shown for cell lines in which a synergism was observed

	Cisplatin alone (ng platinum/10^6 cells)			Cisplatin + IFNγ (ng platinum/10^6 cells)		
	IC_{20}	IC_{50}	IC_{80}	IC_{20}	IC_{50}	IC_{80}
OVCCR1	6.1 ± 0.5	14.4 ± 1.5		8.3 ± 1.5	16.6 ± 2.1	
NIHOVCAR3		5.3 ± 0.3	9.1 ± 1.5		4.9 ± 0.6	7.9 ± 1.2
2008		4.1 ± 0.5	8.2 ± 1.5		4.2 ± 0.8	7.5 ± 1.5
2008/C13*		14.1 ± 1.7	20.1 ± 2.1		14.3 ± 1.2	19.8 ± 1.6

Cells were exposed for 1 h to cisplatin IC_{20}, IC_{50} or IC_{80} in the presence or absence of IFNγ concentrations defined by the fixed ratio of concentrations used for median effect analysis. Values are the means of three independent determinations. Mean ±S.D. are given.

and SKOV3. These two observations (synergism in the cisplatin-resistant cell line and no synergism in the IFNγ-insensitive cell line) support the hypothesis that it is IFNγ which enhances the activity of cisplatin (Table 3).

Interactions between cytotoxic drugs and IFNγ have been previously described. Synergism or additivity have been observed between IFNγ and 5-fluorouracil, doxorubicin, vincristine or vinblastine in different types of human cancer cell lines [21]. Synergism or additivity amplification of the antiproliferative activity of cisplatin and IFNγ have been demonstrated by Marth and colleagues [12] in two out of three cell lines tested, but the mechanism by which IFNγ enhances sensitivity to cisplatin remains undefined. Different hypotheses have been proposed to explain the potential modulation of cisplatin activity or resistance, recently reviewed by Timmer-Boscha and colleagues [22]. Firstly, impaired cisplatin uptake is a common characteristic of cells selected for cisplatin resistance, but we have demonstrated that IFNγ does not produce an increase in cisplatin uptake. Such a mechanism has been described for some drugs such as forskoline [23] or dipyridamole [24], which increase cisplatin cytotoxicity in human ovarian cell lines, sensitive or resistant to cisplatin, by increasing cellular cyclic adenosine monophosphate (c-AMP) levels. Secondly, cellular cisplatin sensitivity is known to be influenced by many other factors (increased glutathione content or enhancement of DNA repair synthesis [25]) which might be modulated by IFNγ. Finally, there are several lines of evidence suggesting that oncogenes might play a role in cisplatin cytotoxicity, even if there is not yet a biochemical explanation for the effects of oncogenes on cisplatin cytotoxicity [26]. Currently, it is well known that IFNγ induces the expression of more than 20 genes and downregulates many others [27, 28]. However, potential interactions at this molecular level seem to be still speculative.

Our findings indicate that, *in vitro*, the maximum potentiation of cisplatin cytotoxicity occurs at concentrations of IFNγ clinically achievable. Both these two agents have exhibited antitumoral activity against human ovarian cancer xenografts growing as ascites or bulky solid tumours in nude mice, when administered i.p. [3, 29]. Using these conditions, we plan to study the *in vivo* combination of cisplatin and IFNγ administered i.p. against human ovarian xenografts which have been established from the cell lines in which we have demonstrated *in vitro* positive interactions. If these animal experiments confirm the *in vitro* data, they should constitute the ultimate step before the introduction of cisplatin–IFNγ combinations in clinical practice.

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H7, an Inhibitor of Protein Kinase C, Inhibits Tumour Cell Division in Mice Bearing Ascitic Ehrlich's Carcinoma

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We have previously shown that H7, an inhibitor of protein kinase C (PKC), inhibits proliferation of several cell lines as well as of primary cultured cells from human tumours. The aim of this work was to assess whether H7 is able to prevent the division of tumour cells in mice bearing Ehrlich's ascitic carcinoma. The LD_{50} of H7 injected intravenously was 61 mg/kg and 94 mg/kg for starved and fed mice, respectively. Acute intraperitoneal injection of 100 mg/kg of H7 decreased the number of mitoses in tumoral cells from ascitic fluid of mice bearing the carcinoma. The reduction was maximal (approximately 50%) after 90 min and then the number of mitosis rose due to a decrease in H7. Continuous delivery of H7 from miniosmotic pumps implanted on the backs of the mice reduced the number of mitoses by approximately 65%, and the effect was maintained for approximately 24 h. The effect cannot be maintained for longer because H7 is unstable at body temperature. These results indicate that inhibition of PKC can block division of tumour cells in carcinoma-bearing animals, and support the idea that inhibitors of PKC could be useful for the clinical control of proliferation of certain tumours.

Key words: protein kinase C, H7, Ehrlich's carcinoma, cell division, tumour proliferation, ascites
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INTRODUCTION

PROTEIN KINASE C (PKC) plays an important role in transmembrane signal transduction and in the control of many cellular responses, including cell proliferation and differentiation, gene expression and tumour promotion [1]. The involvement of PKC in the control of cell growth was initially suggested by the finding that PKC constitutes the receptor for the mitogenic phorbol esters, which are tumour promoters [2]. The role of PKC in the regulation of cellular growth is also supported by the fact that certain growth factors mediate their mitogenic effects in part through a cascade of phosphatidyl inositol hydrolysis and activation of PKC [3, 4]. It has also been shown that PKC activity is overexpressed in aflatoxin-transformed cells [5]. Moreover, PKC activity is increased in human tumour carcinoma and adenoma [6], and the expression of different isoforms of PKC is altered in human astrocytomas/brain tumours, melanomas and