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Potentiation of Tumour Necrosis Factor-mediated Cell Killing by VP16 on Human Ovarian Cancer Cell Lines. *In vitro* Results and Clinical Implications

Monica Valenti, Guido Cimoli, Silvio Parodi, Gabriella Lucia Mariani, Marco Venturini, PierFranco Conte and Patrizia Russo

Synergism between recombinant human tumour necrosis factor (rHuTNF) and DNA topoisomerase II inhibitor VP16 during the killing of cells has been studied in six human ovarian cancer cell lines (A2774, A2780, SW626, IGROV-1, SKOV3, Pa1) and a cervical carcinoma cell line (Me180). Studies were performed using an assay of colony formation inhibition (drug treatment for 1 h) and a growth inhibition assay (continuous exposure for 20 h). Concomitant treatment of cells with VP16+rHuTNF enhanced cell killing in all the cell lines tested—an effect observed in both short- and long-term cytotoxicity assays. This study suggests that the activity of VP16 in ovarian cancer cell lines might be enhanced by rHuTNF in *in vitro* models.
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INTRODUCTION

TUMOUR NECROSIS FACTOR (TNF) was originally described as a polypeptide producing acute and haemorrhagic necrosis of tumour nodules 24–28 h after local or systemic injection in experimental animals [1]. However, this cytokine has a broad spectrum of biological activities including osteolysis, collagenase secretion and angiogenesis [2, 3]: it also plays an important role in the pathophysiology of such diverse conditions as cachexia, endotoxaemia, malaria, arthritis, alcoholic hepatitis and acute respiratory distress syndrome [4–7]. *In vitro* studies indicate both cytostatic and cytotoxic effects against a wide range of human tumour cells [8, 9]. However, TNF is cytotoxic in less than one-third of cell lines tested [10].

A synergistic cytotoxic effect has been reported in cell lines exposed to combinations of TNF and DNA topoisomerase II-targeted anti-cancer drugs, such as etoposide (VP16) and mitoxantrone, *in vitro* [11–14] and *in vivo* [15–17]. In contrast to

Correspondence to P. Russo.
P. Russo, M. Valenti, G. Cimoli, S. Parodi, G.L. Mariani and M. Venturini are at the Department of Chemical Carcinogenesis-Istituto Nazionale per la Ricerca sul Cancro, V. le Benedetto XV, 10 I-16132 Genova; and P.F. Conte is at the Ospedale Santa Chiara, Pisa, Italy.
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these results TNF was unable to enhance the cytotoxicity of VP16 in nine human lung cancer cell lines [18].

Eukaryotic topoisomerase II is an essential nuclear enzyme that is involved in central processes concerning nucleic acid metabolism, including replication, transcription and chromosome segregation [19]. Besides the normal cellular function, topoisomerase II has been shown to act as the primary target for various classes of antineoplastic agents [19]. These drugs are able to stabilise DNA-topoisomerase II complexes which can be detected by the appearance of protein-associated DNA strand breaks.

In this study we have evaluated the cytotoxic activity of recombinant human TNF (rHuTNF) in combination with VP16, a non-intercalating topoisomerase II-targeted drug, on a panel of six human ovarian cancer cell lines and a human cervical carcinoma cell line. Cytotoxicity was determined by either inhibition of colony formation (ICFA) or growth inhibition assays (GIA). The clinical relevance of this drug combination is considered.

MATERIALS AND METHODS

Cells

SW626, A2774, IGROV1 cells (a gift from Dr S. Ferrini, Istituto Nazionale per la Ricerca sul Cancro, Genova), Pa1, Me180 and SKOV3 cells (American Type Culture Collection, Rockville, Maryland) were maintained as monolayer cultures in RPMI medium supplemented with 10% heat inactivated fetal calf serum (HI-FCS) and 1% gentamicin. A2780 cells (a gift from Dr A. Mazzoni, Depha Team, Milano, Italy) were maintained as monolayer cultures in RPMI 1640 with 10% FCS and 1% gentamicin.

Drugs

VP16 was purchased from Bristol-Myers Squibb Italia [Sermoneta (LP) Italy]. rHuTNF was obtained from Knoll-BASF (Ludwigshafen, Germany) and was kept as a stock solution (0.1 mg/ml) at −80°C. Specific activity was 8.74×10^6 U/mg protein. Final concentrations of drugs were prepared in RPMI-1640 supplemented with 10% serum immediately prior to use.

Cytotoxicity assay

Growth inhibition assay (GIA). Cells were plated into 96-well flat-bottomed microtitre plates at a seeding density of 3×10^4 cells per well in a volume of 0.1 ml. Cells were incubated for 24 h at 37°C. Medium was then replaced with 0.2 ml of fresh medium containing VP16 and/or rHuTNF. Cells were then incubated at 37°C for a further 20 h.

Cytotoxicity was monitored using the crystal violet assay [20] with minor modifications [11].

The IC_{50} (50% inhibitory concentration) was calculated with a linear interpolation in the interval of concentrations with a cellular survival immediately above and below 50%.

Inhibition of colony formation assay (ICFA). The assay was performed according to Holm *et al.* [21]. Briefly, following drug treatment (1 h at 37°C with various concentrations of VP16 or rHuTNF or with various concentrations of VP16 + 1000 U/ml rHuTNF), cells were suspended in medium, washed three times with phosphate buffered saline (PBS) and then counted. 500 cells were seeded in triplicate into 60 cm^2 plates in 5 ml of medium for each treatment condition. Cell cultures were incubated for 5–7 days, after which they were washed twice with 5 ml PBS. Colonies were fixed with 95% methanol (15 min), stained with methylene blue (20 min), and counted. The plating efficiency of drug-treated cells was divided by the plating efficiency of untreated cells, to yield a "survival fraction". The plating efficiency for untreated cells was 80–95%. The IC_{50} was calculated as reported above.

RESULTS

Potentiation of rHuTNF-mediated cell killing by VP16

Growth-inhibition assay (GIA). The effects of rHuTNF, at equal concentrations determined by rHuTNF dose–response curves (data not shown), on cytotoxicity of VP16 were studied in all cell lines. Table 1 shows the VP16 IC_{50} values obtained in the presence or absence of rHuTNF. A2780 cells exhibited the highest degree of sensitivity to VP16 alone (IC_{50} = 0.42 μmol/l), whereas SW626 cells presented the highest degree of resistance (IC_{50} = 119.0 μmol/l). A significant effect of rHuTNF (potentiation rate > 10, expressed as a ratio of the VP16 IC_{50} with and without rHuTNF) on the cytotoxicity of VP16 was observed in five lines (A2780, A2774, Pa1, IGROV-1, SKOV-3). A less marked effect (potentiation rate > 5) was observed in two additional lines (SW626, Me180).

The dose–response curve for VP16 is shown in Fig. 1 (a–f) for five ovarian cancer cell lines and one cervical carcinoma cell line, (we have previously demonstrated a strong potentiation effect of VP16 by rHuTNF on the A2774 cell line, [12]). Over the dose range tested, VP16 treatment had a significant dose-related cytotoxic effect after a 20-h incubation period in all cell lines tested. The effects of co-supplementation of medium with rHuTNF in addition to VP16 are also shown in Fig. 1. When rHuTNF was added at equitoxic concentration (the equal amounts of dose of rHuTNF were calculated by interpolating those dosages of rHuTNF giving the same survival fractions as the dosages of VP16 used), a significant enhancement of cytotoxicity was seen in all cell lines. This enhancement of efficacy cannot be a simple additive effect, since the effect was much greater than the curve extrapolated for a double concentration of VP16 alone (see Fig. 1).

Inhibition of colony formation assay (ICFA). One-hour treatment with rHuTNF alone (from 10 to 1000 U/ml) did not

Table 1. Relative cytotoxicity of VP16 on six ovarian cancer cell lines and one cervical carcinoma cell line (Me180) and effect of rHuTNF on the sensitivity to VP16, evaluated after continuous exposure (20 h) in the growth inhibition assay

Cell line	IC_{50} VP16 (μmol/l) −rHuTNF	IC_{50} VP16 (μmol/l) +rHuTNF*	rHuTNF P.R.†
A2780	0.42 ± 0.02	0.0025 ± 0.008	168.0
A2774	28.90 ± 5.3	0.59 ± 0.04	49.0
PA1	0.29 ± 0.05	0.01 ± 0.007	29.0
IGROV-1	0.19 ± 0.03	0.01 ± 0.005	19.0
SKOV-3	0.24 ± 0.02	0.02 ± 0.001	12.0
SW626	119.00 ± 10.50	12.60 ± 1.4	9.4
Me180	0.10 ± 0.05	0.015 ± 0.006	6.7

Values represent the average of at least three independent determinations. Means ± S. E. are given.

IC_{50} = 50% inhibitory concentration.

* rHuTNF concentration was equitoxic with respect to VP16 dosages and was added simultaneously to VP16.

P.R. = potentiation ratio.

† Degree of potentiation of each different line by rHuTNF expressed as ratio of the VP16 IC_{50} without and with rHuTNF.

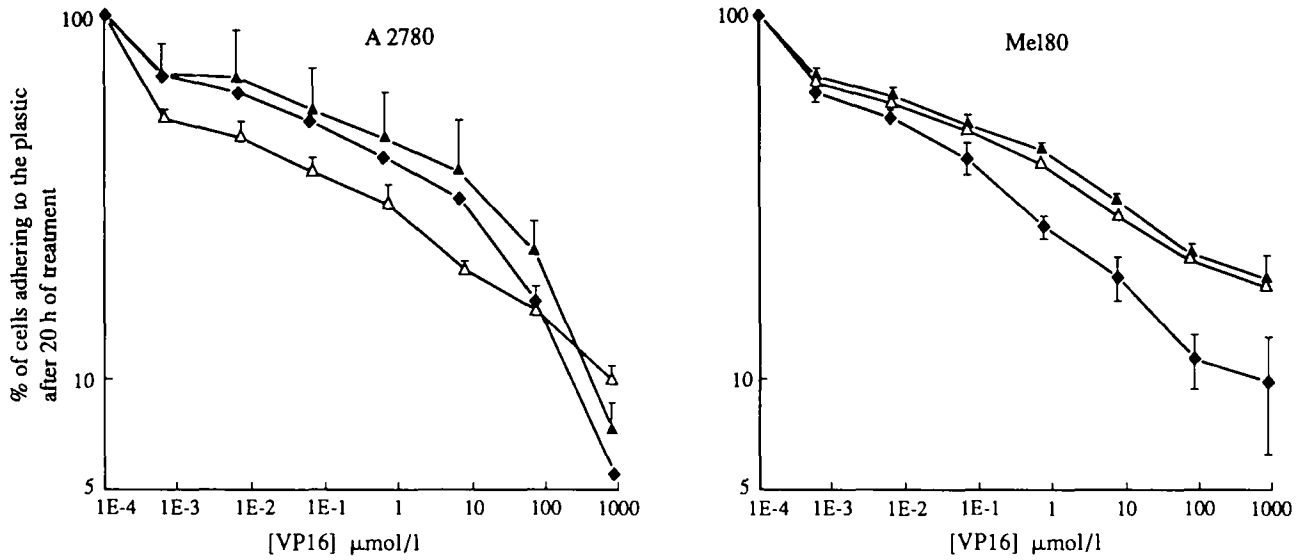

Fig. 1. Effect of rHuTNF on the cytotoxicity of VP16 on six ovarian cancer cell lines and on one cervical carcinoma cell line (Mel80). GIA assay. (a) Effect of VP16 and rHuTNF on SW626 ovarian cancer cell line. ▲ = SW626 cell line exposed to various concentration of VP16. △ = SW626 cell line, extrapolated effect with a double concentrations of VP16. ◆ = SW626 cell line VP16 and rHuTNF at equitoxic concentration. The equitoxic dosages are the following: (1) = 2.29 U/ml; (2) = 8.13 U/ml; (3) = 27.5 U/ml; (4) = 169.8 U/ml; (5) = 288.4 U/ml. (b) Effect of VP16 and rHuTNF on IGROV-1 ovarian cancer cell line. As in (a). The equitoxic dosages of rHuTNF are the following. (1) = 158.5 U/ml; from (2) to (7) = 1000 U/ml. 1000 U/ml were given when it was impossible to calculate the equitoxic dosages. (c) Effect of VP16 and rHuTNF on PA-1 ovarian cancer cell line. As in panel (a). The equitoxic dosages of rHuTNF are the following: (1) = 0.56 U/ml; (2) = 25.1 U/ml; from (3) to (7) = 1000 U/ml. (d) Effect of VP16 and rHuTNF on SKOV3 ovarian cancer cell line. As in (a). The equitoxic dosages of rHuTNF are the following: (1) = 0.31 U/ml; (2) = 67.6 U/ml; from (3) to (7) = 1000 U/ml. (e) Effect of VP16 and rHuTNF on A2780 ovarian cancer cell line. As in (a). The equitoxic dosages of rHuTNF are the following: (1) = 1.15 U/ml; (2) = 1.48 U/ml; (3) = 4.7 U/ml; (4) = 14.1 U/ml; (5) = 59 U/ml; (6) and (7) = 1000 U/ml. (f) Effect of VP16 and rHuTNF on Mel80 cervical carcinoma cell line. As in (a). The equitoxic dosages of rHuTNF are the following: (1) = 4.57 U/ml; (2) = 70.8 U/ml; from (3) to (7) = 1000 U/ml.

produce significant cytotoxicity in the cell lines tested (data not shown). At the highest concentration, rHuTNF (1000 U/ml) exhibited less than 4% cytotoxicity in the 1-h assay; further experiments of drug synergy, using the ICFA method, were carried out using 1000 U/ml of rHuTNF. When rHuTNF was simultaneously incubated with different concentrations of VP16, a significant inhibition of colony formation was observed in all cell lines and representative dose–response curves are shown in Fig. 2. Table 2 shows the IC_{50} values obtained with VP16 in the presence or absence of rHuTNF. According to the previous results, A2780 cells exhibited the highest degree of potentiation of VP16-mediated cytotoxicity.

DISCUSSION

Ovarian cancer is the third most frequent cause of death from cancer in women and the leading cause of death among gynaecological malignancies. The use of aggressive debulking surgery followed by cisplatin (CDDP)-based regimens achieves a high overall response rate of up to 80%, with clinical complete responses (CR) in approximately 40–50% of patients. Despite these high CR rates, the number of patients with pCR (pathological), assessed by laparatomy, is only 15–20% [22, 23]. Therefore, new therapeutic modalities, such as intraperitoneal (i.p.) chemotherapy, consolidation treatments or effective biological response modifiers (BRM), are required in order to effectively improve outcome.

The natural tendency of ovarian cancer to spread and to remain confined for a long time within the abdominal cavity makes this tumour particularly suitable for the intraperitoneal route of chemotherapy [24]. This approach allows higher drug concentrations to be reached in the tumour, but with low serum levels and hopefully lower toxicity.

Recently, a phase I study [25] of intraperitoneal VP16, administered in patients suffering from cancer confined to the abdominal cavity, estimated the relative pharmacological advantage [area under curve (AUC) in peritoneal cavity divided by AUC in systemic circulation] at 2.8.

Significant improvements in experimental cancer therapies have been made by combining BRM and cytotoxic drugs in order to augment their effectiveness and reduce the required doses. Furthermore, *in vivo* studies in mice have demonstrated a clearcut advantage of intraperitoneal therapy with BRM compared with the systemic route of administration [1]. An improvement of the *in vivo* (murine tumour models) antitumour effects was seen with combined administration of rHuTNF with VP16 [15, 17] in bladder tumours. The combination of TNF + VP16, evaluated in a human renal carcinoma xenograft, caused increased tumour inhibition in respect to VP16 alone [16]. *In vitro* studies demonstrated that rHuTNF augments the cytolytic activity of topoisomerase II-directed chemotherapeutic drugs and that this combination of agents was effective in reducing tumour volume *in vivo* [15]. Unfortunately, many topoisomerase II inhibitors, with the exception of VP16 and mitoxantrone, are not suitable for intraperitoneal administration, because of their local toxicity.

Recently, we have demonstrated that rHuTNF potentiates mitoxantrone-cytotoxicity in six human ovarian cancer cell lines [13]. Thus we extended our research to studying the activity of a combination of rHuTNF + VP16 in a panel of six ovarian cancer cell lines.

Our findings indicate that rHuTNF significantly increases the cytotoxic activity of VP16 in such cell lines and in one cervical carcinoma cell line, at rHuTNF concentrations that can be achieved clinically ($IC_{50} < 5000$ U/ml) [26].

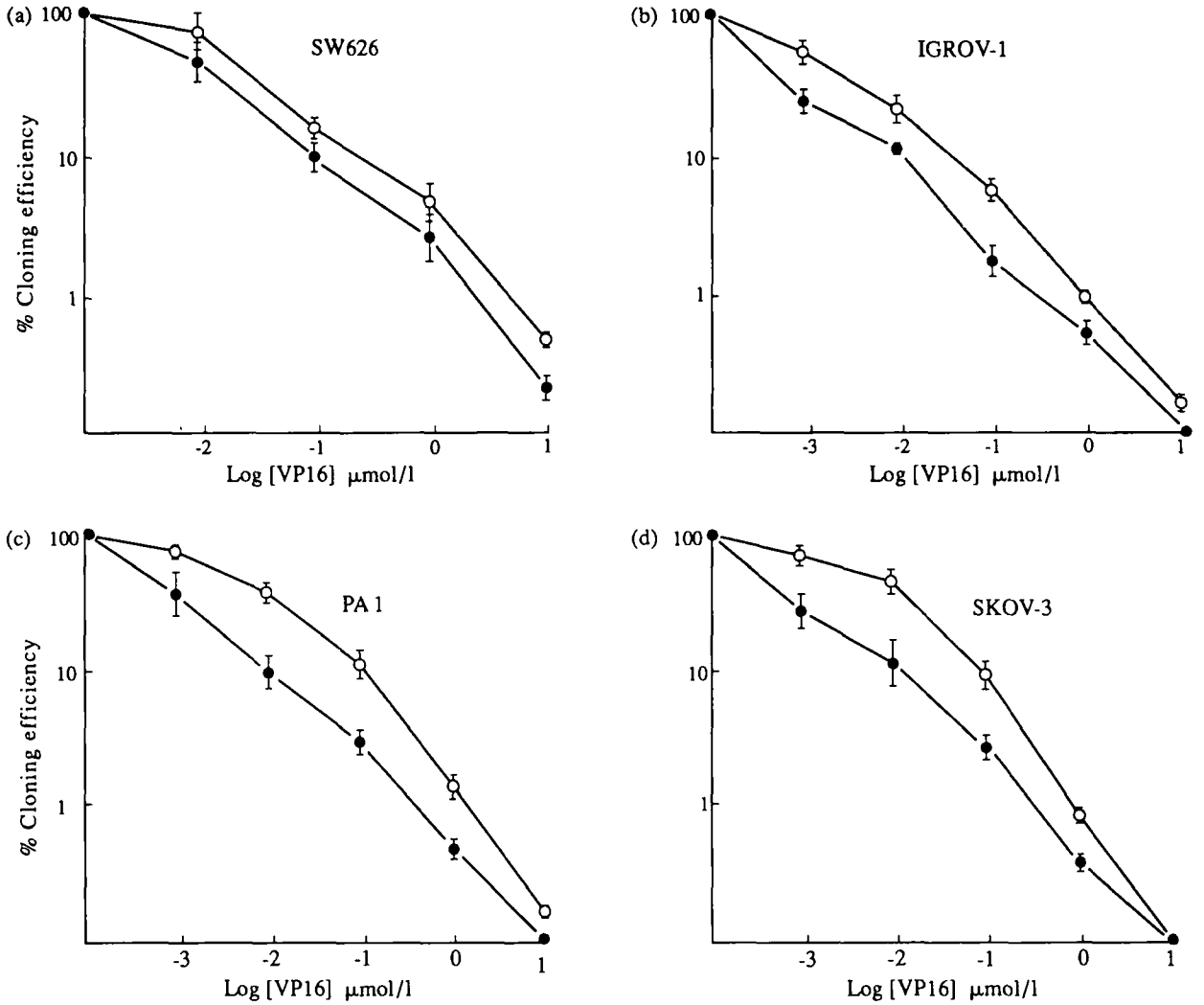

Fig. 2. Representative curves depicting the effect of VP16 on the cytotoxicity of rHuTNF in four human ovarian cancer cell lines (SW626, IGROV-1, PA1 and SKOV3) as determined by the ICFA assay. Cells were exposed to different concentrations of VP16 for 1 h at 37°C in the presence or absence of 1000 U/ml of rHuTNF. ○ = VP16. • = VP16 + rHuTNF.

Table 2. Relative cytotoxicity of VP16 on six ovarian cancer cell lines and one cervical carcinoma cell line (Me180) and effect of rHuTNF on the sensitivity to VP16, evaluated after 1 h exposition in the inhibition of colony formation assay

Cell line	IC_{50} VP16 μmol/l −rHuTNF	IC_{50} VP16 μmol/l +rHuTNF*	rHu TNF P.R.†
A2780	0.13 ± 0.04	0.013 ± 0.007	10.0
A2774	38.30 ± 2.30	7.94 ± 2.6	4.8
PA1	0.32 ± 0.05	0.08 ± 0.006	4.0
IGROV-1	0.19 ± 0.05	0.05 ± 0.002	3.8
SKOV-3	0.22 ± 0.01	0.07 ± 0.004	3.1
SW626	132.40 ± 12.50	71.90 ± 10.4	1.8
Me180	0.13 ± 0.04	0.07 ± 0.003	1.9

Values represent the average of last three independent determinations. Means ± S. E. are given.

IC_{50} = 50% inhibitory concentration.

* rHuTNF 1000 U/ml was added simultaneously to VP16.

P.R. = potentiation ratio.

† Degree of potentiation of each different line by rHuTNF expressed as ratio of VP16 IC_{50} without and with rHuTNF.

It is important to note that rHuTNF potentiates VP16 cytotoxicity not only after continuous exposure to the drug (20 h) but also after 1 h of incubation. When the combination of VP16 + rHuTNF is given for 20 h (Table 1), an approximately 10-fold potentiation effect was seen relative to 1-h administration (Table 2).

This could be due, in part, to the methodological differences in the measurement of the effects (GIA versus ICFA). However, the difference could also arise from a more reversible effect induced after 1-h treatment in respect to 20 h. After prolonged treatment with both TNF [9] and VP16 [27] apoptotic phenomena and their potentiation are more likely to have been induced. Working with human lung cancer cell lines Giaccone *et al.* [18] observed no significant increase in VP16 cytotoxicity (20-h assay) by TNF; and Doyle *et al.* [28], also working with human lung cancer cell lines, reported that the interaction of TNF with VP16 varied between cell lines and between subclasses of human lung cancers (weak potentiation or protective effects of VP16 cytotoxicity). In contrast, Donaldson *et al.* [16] working with human renal cell carcinomas *in vivo*, Das *et al.* [17] working with human bladder transitional cell carcinomas *in vivo* and Alexander *et al.* [15] working with murine bladder cancers *in vivo* and *in vitro*, reported a marked potentiation of the antineoplastic effects

of VP16 by TNF. It is possible that the interaction of TNF and drugs targeted by DNA topoisomerase II varied not only between subclasses of cell lines but also between cells derived from different tissues. Recently, Mutch *et al.* [29] reported that resistance to cytolysis by TNF in malignant gynecological cell lines is associated with the expression of proteins that prevent the activation of phospholipase A2 by TNF. However, they suggest that these cells become sensitive to cytolysis by TNF in the presence of protein synthesis inhibitors such as actinomycin D, which interestingly is also a topoisomerase II inhibitor [19]. Also in our experience, working either on human ovarian cancer cell lines [11–13] or on cells obtained from fresh ascites from patients with ovarian cancer [14], we obtained a partial response to TNF alone but a marked potentiation of the cytotoxic effects of VP16, mitoxantrone or doxorubicin by TNF. In combining TNF and topoisomerase II inhibitors the sequence of exposure appears to be crucial for synergistic antiproliferative interactions.

In this work we have extended our previous observation with mitoxantrone [13] to VP16. It is possible that the potentiation effect between rHuTNF and VP16 (or mitoxantrone) is a general property of all topoisomerase II inhibitors in ovarian cell lines. These data may be relevant for future planning of animal and/or human trials using rHuTNF and topoisomerase II-targeted drugs for the treatment of cancer.

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