Anti-Cancer Drugs 1999, **10**, pp. 195–201

Research paper

Cellular pharmacology of the combination of oxaliplatin with topotecan in the IGROV-1 human ovarian cancer cell line

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The clinical development of combinations of cisplatin or carboplatin with DNA topoisomerase I (Topo I) inhibitors is based on their overlapping spectrum of antitumor activity and their *in vitro* synergy, but is limited by significant hematotoxicity. We studied the cellular interactions between oxaliplatin and topotecan in the IGROV-1 human ovarian cancer cell line prior to evaluating the combination in the clinic. Growth inhibition was studied after a 96 h exposure to oxaliplatin and topotecan. The analysis of the cytotoxicity by the isobolograms method revealed supra-additivity with maximal cytotoxicity obtained by giving oxaliplatin prior to topotecan. In the presence of topotecan, the formation of oxaliplatin-induced DNA interstrand crosslinks was not modified in cells, but their reversion was slower, as measured by alkaline elution. Successive topotecan exposures did not affect the level of Topo I-mediated DNA single-strand breaks (SSBs). Pre-exposure to oxaliplatin transiently increased Topo I-mediated SSBs, suggesting that DNA platination might stimulate Topo I DNA cleavage activity. Hence, the cellular pharmacology of oxaliplatin combined with topotecan appeared highly dependent on the schedule. Therefore, this study suggests that the combination of topotecan with oxaliplatin might exhibit sequence-dependent pharmacodynamic interactions in the clinic. [© 1999 Lippincott Williams & Wilkins.]

Key words: Chemotherapy, DACH platinum, DNA damage, DNA topoisomerase I, topotecan, synergism.

Introduction

Several new cytotoxic agents with original mechanism of action have recently been demonstrated active in second-line therapy after a cisplatin-containing regimen in advanced ovarian cancer patients.[1,2] The inclusion of these agents into chemotherapeutic combinations is presently a priority to improve the prognosis of recurrent ovarian cancer patients.

One of these agents is topotecan, already approved for the treatment of refractory ovarian cancer. Topotecan has been found as active as paclitaxel in cisplatin-pretreated ovarian cancer patients.[3] Topotecan is a camptothecin derivative and specifically inhibits DNA topoisomerase I (Topo I) by stabilizing a covalent Topo I-DNA complex (reviewed elsewhere[4–7]). Collisions between the ternary cleavable complexes and advancing replication forks result in replication fork arrest and fork breakage. Such a mechanism may account for the lethal effect of topotecan observed *in vivo*. The combination of topotecan with other DNA damaging agents, such as alkylating agents or cisplatin, is synergistic *in vitro* but may depend on the schedule.[8–12]

Another active agent in advanced ovarian cancer patients is oxaliplatin [*trans*-l-1,2-diaminocyclohexane oxalato Pt(II); oxaliplatin]. A 29% objective response rate as single agent in 31 evaluable heavily pretreated ovarian cancer patients has been reported, with some evidence of efficacy in platinum refractory patients.[13] Oxaliplatin is a DACH carrier ligand-based platinum (reviewed elsewhere[14–16]). Similarly to cisplatin and carboplatin, the cytotoxicity of oxaliplatin is a consequence of the formation of DNA intra- and interstrand crosslinks.[14,16] However, oxaliplatin adduct structure differs from both cisplatin and carboplatin.[17] The rate of monoadduct to diadduct conversions and the resulting DNA distortion produced by cisplatin and oxaliplatin might explain the efficacy of oxaliplatin in mismatch-repair-deficient cells,[18–20] and its lack of cross-resistance with cisplatin and carboplatin both *in vitro*[21] and in the clinic.[13] Both differences in DNA damage and DNA damage

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signaling might account for its antitumor activity in cisplatin-resistant cancer cells.[17,19-21] Because of these differences in DNA damage and DNA repair, it remained uncertain whether oxaliplatin would exhibit similar behavior to cisplatin *in vitro* in combination with other compounds.

Combinations of topotecan with any DNA damaging agent is expected to be supra-additive and its association with oxaliplatin appears in this regard of particular interest in advanced ovarian cancer patients. This *in vitro* study examined whether topotecan and oxaliplatin exhibit cellular interactions in the human ovarian cancer cell line IGROV-1. Cellular interactions were found between oxaliplatin and topotecan resulting in supra-additive cytotoxicity. As a consequence of this *in vitro* potentiation, a pharmacodynamic interaction is expected in the clinic with this new combination, with higher toxicity giving oxaliplatin prior to topotecan.

Material and methods

Drugs and chemicals

Oxaliplatin and topotecan were provided by the P Brousse hospital pharmacy. Stock solutions of topotecan were prepared in DMSO at 10 mM. Oxaliplatin was prepared extemporaneously for each experiment and diluted in 5% dextrose immediately prior to use.

Cell culture

IGROV-1 human ovarian adenocarcinoma cells were provided by Dr Pommier (NCI, NIH, Bethesda, MD), and were grown in monolayer cultures in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum and 2 mM glutamine. No antibiotic was added to the medium. The cells were trypsinized and passed twice a week.

Cytotoxicity assays

We used the (4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO) dye (MTT) assay previously described[22] to evaluate the growth inhibitory effects of the cytotoxic agents. Cell suspensions containing 2×10^4 viable cells/ml were seeded into 96-well microtiter plates. After a 48 h incubation, the cells were treated for 96 h with various concentrations of topotecan (10^{-10} to 10^{-5} M) and oxaliplatin (10^{-10} to 10^{-5} M). In the sequential schedule, topotecan was given 24 h prior to or after oxaliplatin. Following exposure to the drugs, 50 μl of 125 μM MTT solution was added to each well and the plates were incubated at 37°C for another 3 h to allow MTT metabolization into formazan crystals. The formazan crystals were finally solubilized adding 50 μl of 25% sodium dodecylsulfate (SDS) solution (Sigma) into each well. Absorbance at 540 nm was measured using a Delta Soft ELISA analysis program for a Macintosh computer interfaced with a Bio-Tek microplate reader (EL-340; BioMetallics, Princeton, NJ). Wells containing only RPMI 1640-fetal bovine serum and MTT were used as controls. Each experiment was performed using eight replicated wells for each drug concentration and carried out independently at least 3 times. The IC_{50} was defined as the concentration that reduced the absorbance in each test by 50%. The absorbance was calculated as (mean absorbance of eight wells containing drug − absorbance of eight control wells)/(mean absorbance of eight drug-free wells − absorbance of eight control wells) × 100.

Analysis of combination effects

Combination analysis was performed using the method described by Chou and a software program for automated analysis.[9,23] The influence on the combination of the two drugs was evaluated in comparing the sequential assays with assays involving only oxaliplatin or topotecan exposures alone. The combination effect was evaluated from iso-effect analysis combination indexes (CI) calculated as following: $CI=(C)_{oxaliplatin}/(Cx)_{oxaliplatin} + (C)_{topotecan}/(Cx)_{topotecan}$; $(Cx)_{oxaliplatin}$ and $(Cx)_{topotecan}$ being the concentration needed, respectively, with oxaliplatin and topotecan alone to achieve a given effect (x%), $(C)_{oxaliplatin}$ and $(C)_{topotecan}$ being the concentration, respectively, of oxaliplatin and topotecan needed for the same effect (x%) when the drugs were combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The combination was considered as positive (synergistic) when the combination index was lower than 1 and negative (antagonistic) when was greater than 1.

Measurement of DNA interstrand crosslinks (ISCs)

ISCs were measured using alkaline elution techniques as described previously.[24] DNA ISCs were assayed after 300 rad irradiation of the cells. After the irradiation, the filter elution assay was done as described below

for the measurement of DNA single-strand breaks (SSBs). The relative ISC frequency (in rad-eq) can be obtained by the formula: $ISC=\{[(1-R_0)/(1-R_1)]^{1/2}-1\} \times 300$, where 300 represents the standard irradiation dose (3 Gy = 300 rad) used to introduce random SSBs, R_0 represents the DNA retention of control cells treated only with the standard irradiation at a fixed elution end-point, and R_1 represents the DNA retention of experimental cells treated with oxaliplatin and the standard dose of radiation at the same fixed elution endpoint.

Measurement of Topo I-mediated DNA SSBs

The alkaline elution procedures were described previously.[24] DNA SSBs were measured by alkaline elution. IGROV-1 experimental cells and control cells were labeled with 0.02 μCi/ml [methyl-^{14}C]thymidine (Dupont/NEN, Boston, MA) for 48 h and with 0.2 μCi/ml [methyl-^{3}H]thymidine for 24 h, respectively. Radiolabel was chased by resuspension in non-radioactive fresh medium overnight. Following 30 min topotecan treatment at 37°C in culture medium, cells were washed and scraped with ice-cold Hanks' balanced salt solution that contained the same topotecan concentration to avoid DNA damage reversal. Samples were loaded onto polycarbonate filters (2 μm pore size; Poretics, Livermore, CA) and lysed with 5 ml SDS lysis solution (0.1 M glycine, 2% SDS and 0.025 M Na_2EDTA, pH 10) in the presence of 0.5 μg/ml proteinase K. [Methyl-^{3}H]thymidine-labeled control cells had been irradiated at 2000 rad prior to be loaded. Lysates were rinsed with 5 ml of 0.02 M EDTA (pH 10.0) and elutions were carried out with a mixture of tetrapropylammonium hydroxyde and EDTA and 0.1% SDS (pH 12.1) at a flow rate of 12-16 ml/min. Fractions were collected every 5 min for 30 min. The frequency of SSBs was expressed in rad-eq.

Results

Cytotoxicity of oxaliplatin combined with topotecan

IGROV-1 cells were exposed to either topotecan or oxaliplatin alone or to both drugs, for 1 h. As shown in Fig. 1(A), the concentrations of topotecan and oxaliplatin which inhibited the growth of 50% of the cells (GI_{50}) when given alone for 96 h were 20 and 200 nM, respectively. Greater than additive growth inhibition was observed with the topotecan-oxaliplatin combination (see Fig. 1A). The CI isobologram equation was used for data analysis of the topotecan-oxaliplatin combination and the results are illustrated in Fig. 1(B). These data confirmed with the DACH platinum derivative oxaliplatin the synergy previously described between DNA Topo I inhibitors and cisplatin.[10-12,23-25] A supra-additive effect was found

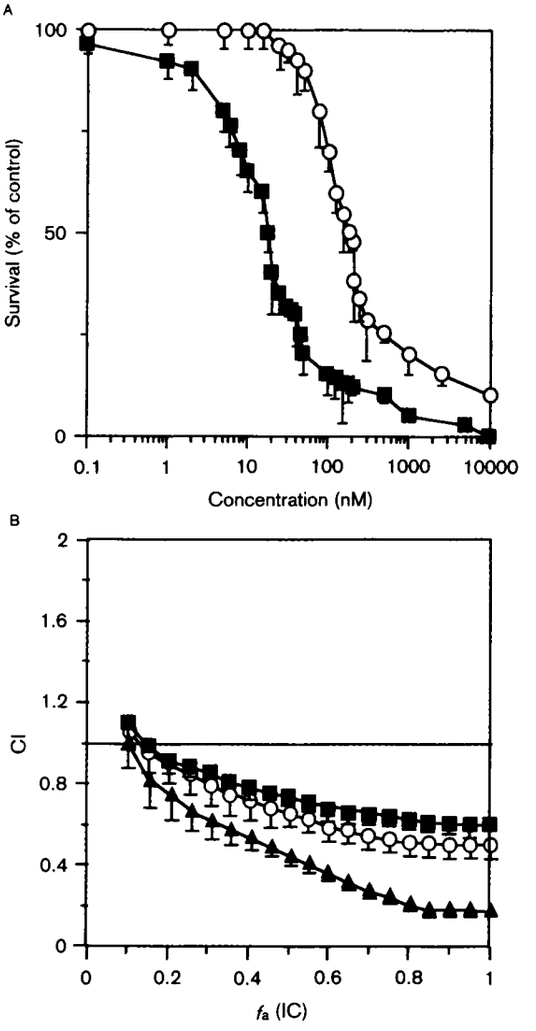

Figure 1. Cytotoxicity after 96 h exposure to oxaliplatin, topotecan or their combination in the IGROV-1 human ovarian cancer cell line. (A) Topotecan (black squares), oxaliplatin (opened circles). Data are means of three to six experiments. Bars, SD. (B) Synergism was studied when oxaliplatin and topotecan were combined, giving topotecan before (black squares), simultaneously (opened circles) or after oxaliplatin (black triangles), using the method described by Chou *et al.*[11,12] CI > 1, antagonism; CI < 1, synergism; CI=1, additive effect.

with either sequence but was maximal when giving oxaliplatin prior to topotecan (Fig. 1B).

The half-life of oxaliplatin-induced DNA ISCs is increased by topotecan

The effect of topotecan on the kinetics of oxaliplatin-induced DNA ISCs was measured in cells by alkaline elution. As expected from a Topo I inhibitor, the exposure to topotecan did not result in any DNA ISC formation. The formation of DNA ISCs was measured after exposure to 50 μM oxaliplatin for 1 h, in the presence or absence of topotecan concentrations ranging from 0.1 to 10 μM. Topotecan did not affect the amount of detectable oxaliplatin DNA ISCs 6 h after drug removal (Fig. 2). However, there was a concentration-dependent effect of topotecan on the reversion of oxaliplatin ISCs (Fig. 3). Using concentrations of topotecan higher than 1 μM topotecan allowed us to detect more persistent levels of ISCs 15 h after drug removal in the combination arm, in comparison to the cells treated with oxaliplatin alone. These results indicated that topotecan slows down the reversion of ISCs.

Oxaliplatin transiently increases topotecan-induced DNA SSBs

We aimed to determine whether Topo I-cleavable complexes might be activated or down-regulated after oxaliplatin-induced DNA damage. The formation of Topo I mediated DNA-protein crosslinks and Topo I-mediated DNA SSBs is associated with a 1:1 ratio of DNA-protein crosslinks/SSB.[4-7] Since oxaliplatin created covalent DNA-protein crosslinks *per se* (data not shown), Topo I-mediated SSBs rather than DNA-protein crosslinks were measured in IGROV-1 cells. The cells were treated for 30 min with topotecan at several times after oxaliplatin treatment, and DNA SSBs were measured by alkaline elution in deproteinizing and denaturating conditions. In the absence of oxaliplatin treatment, repeated 30 min exposures to topotecan induced the same amount of DNA SSBs (Fig. 4A). Similar formation of Topo I-mediated DNA SSBs was found at the end of the 1 h exposure to oxaliplatin. However, 2 h after oxaliplatin removal, a transient but reproducible increase in Topo I-mediated DNA SSBs could be detected (Fig. 4B).

Discussion

We found in this study *in vitro* synergy between topotecan and oxaliplatin. Topotecan increased the half-life of oxaliplatin-induced DNA ISCs, while a transient increase in Topo I-mediated DNA SSBs could be detected after oxaliplatin exposure, both effects leading to increased cytotoxicity of the sequence oxaliplatin followed by topotecan.

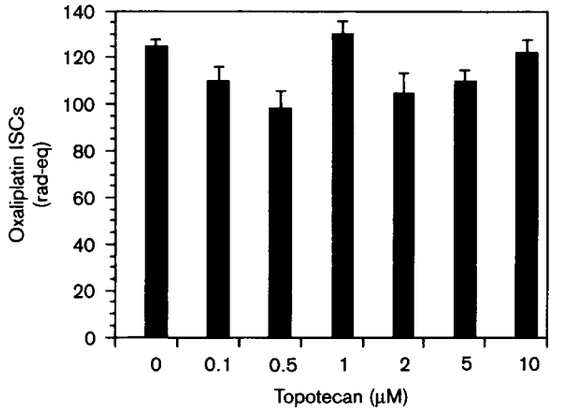

Figure 2. Effect of topotecan on the peak of oxaliplatin-induced DNA ISCs. IGROV-1 cells were exposed for 1 h to 50 μM oxaliplatin or 50 μM oxaliplatin + various concentrations of topotecan. DNA ISCs were measured 6 h after drug removal.

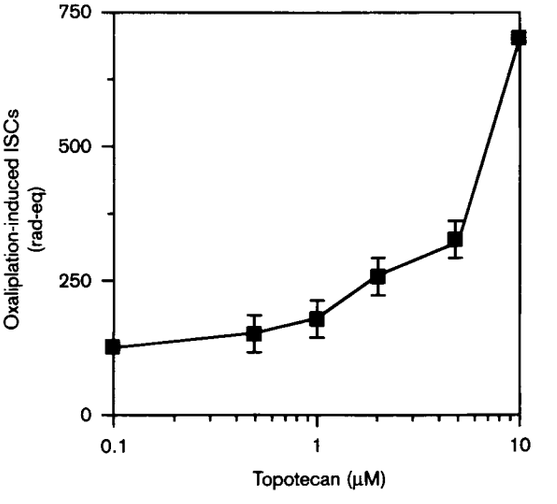

Figure 3. Effect of topotecan on the reversion of oxaliplatin-induced DNA ISCs. IGROV-1 cells were exposed for 1 h to 50 μM oxaliplatin or 50 μM oxaliplatin + various concentrations of topotecan. DNA ISCs were measured 15 h after drug removal.

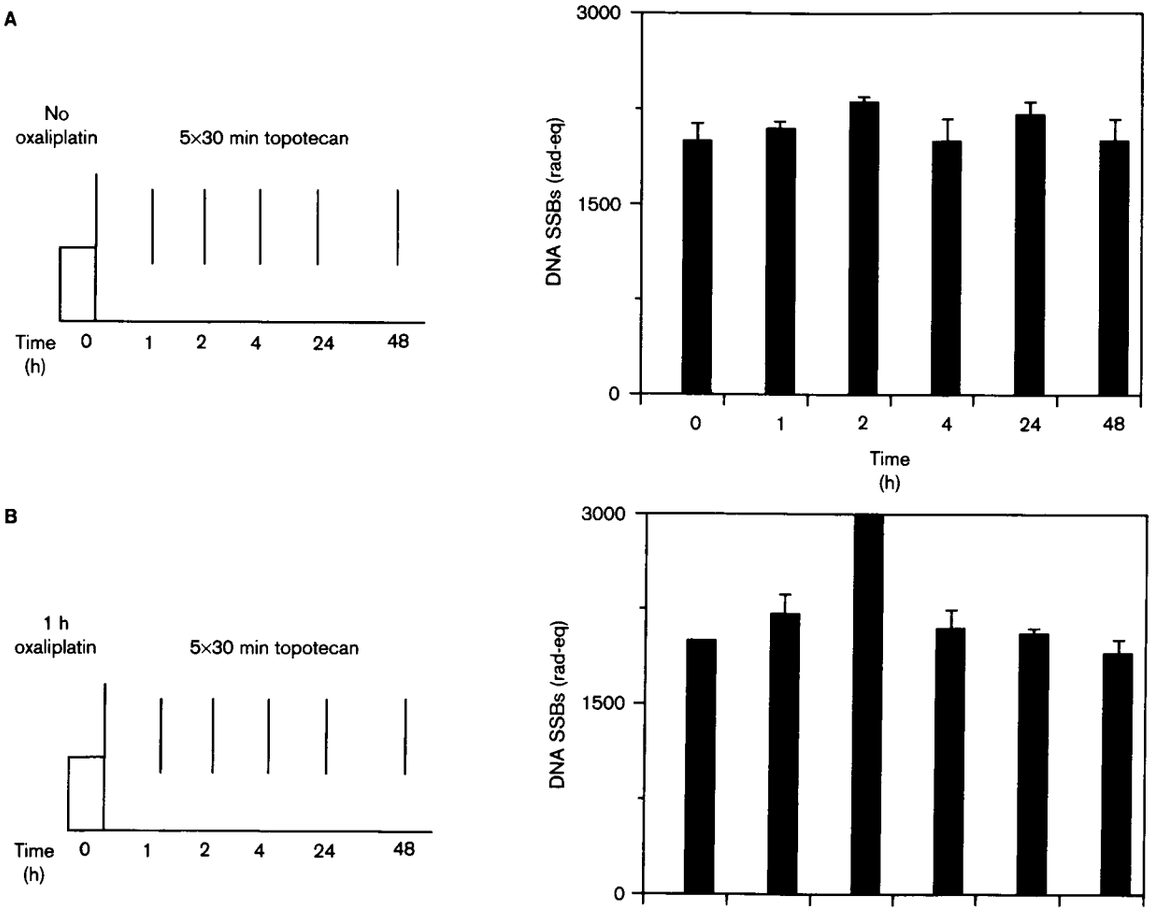

Figure 4. Effect of oxaliplatin on topotecan-induced DNA SSBs. (A) IGROV-1 cells were exposed to 1 μM topotecan for 30 min and again 1–48 h later. (B) IGROV-1 cells were treated with 1 μM topotecan for 30 min at various times ranging from 0 to 48 h following a 1 h exposure to 50 μM oxaliplatin.

Correlations between DNA damage formation and cytotoxicity are rarely found,[26-28] while any effect on the duration of DNA damage is frequently converted in gain in cell killing.[11,12,24] Topo I inhibitors have been found to potentiate the cytotoxicity of DNA damaging agents, such as alkylating agents.[8-10] The combination of Topo I inhibitors with cisplatin has been previously studied both *in vitro* and in the clinical setting. Topotecan,[9-12] SN-38[25] and 9-amino-camptothecin[24] were found to potentiate cisplatin cytotoxicity and to increase the duration of cisplatin-induced ISCs.[11,12,24] Slower reversion of cisplatin ISCs was attributed to the inhibition of DNA and/or RNA synthesis which are associated with Topo I inhibition. The repair of oxaliplatin-induced ISCs requires unscheduled DNA synthesis and the present effect of topotecan on the duration of oxaliplatin ISCs might be the consequence of topotecan-induced DNA synthesis inhibition. Even if the role of Topo I in DNA repair is likely, there is still no direct evidence of Topo I participation in the repair of DNA damage. Several types of DNA damage have been found *in vitro* to stimulate Topo I DNA cleavage activity.[29,30] Abasic sites are DNA lesions produced during excision repair of base damage due to alkylation.[31] The generation of abasic sites and DNA mismatches may alter Topo I DNA cleavage *in vitro*, depending on their position relative to the Topo I cleavage site.[30] To date, no effect of DNA platination on Topo I activity has been reported. We observed here that oxaliplatin DNA damages were able to transiently stimulate Topo I-mediated DNA SSBs and thus might stimulate Topo I DNA cleavage activity. Hence, oxaliplatin-induced DNA damage sensitized the cells

to the toxic effects of topotecan. Both the effect of topotecan on oxaliplatin ISCs reversal and the action of oxaliplatin on the formation of Topo I-mediated SSBs suggest that the sequence of oxaliplatin followed by topotecan should be the most toxic. Consistently, the cytotoxicity of the combination oxaliplatin-topotecan was found higher using this latter sequence.

Several clinical trials combining cisplatin with a Topo I inhibitor have already been achieved. Despite no evidence of pharmacokinetic interaction between cisplatin and CPT-11[32] or minor renal interaction between cisplatin and topotecan, clear sequence-dependent toxicity was observed in patients with the combination beginning with cisplatin always being the most toxic.[32-35] Hence, in agreement with the laboratory findings, marked differences in the toxicity pattern were observed during the clinical evaluation of the combination cisplatin-topotecan,[33-35] depending on the sequence of administration.[35] The administration of cisplatin on day 1 appeared more myelosuppressive than on day 5, topotecan being given as a 30 min infusion on day 1-5.[35] Based on the clinical observations of the combination cisplatin-topotecan and our present study, the combination oxaliplatin-topotecan is expected to be associated with increased hematotoxicity when the platinum derivative is administered before the camptothecin derivative. The combination of topotecan with oxaliplatin appears as an attractive association both because of the synergy between alkylating agents and DNA Topo I inhibitors, and because oxaliplatin exhibits minimal hematotoxicity. A phase I clinical trial is ongoing to evaluate the feasability of this combination in advanced cancer patients. The present *in vitro* study was undertaken to define the optimal scheduling for the combination of topotecan with oxaliplatin and detect any argument for pharmacodynamic potentiation. Evidence of significant cellular interactions between oxaliplatin and topotecan lead us to expect more than additive pharmacodynamic effects between these two agents, even in the absence of any pharmacokinetic interaction. We are presently evaluating in the clinic the feasability of either sequence of the combination of oxaliplatin and topotecan with concomittant measurement of Topo I-cleavable complexes using the previously described ICE bioassay[36] and oxaliplatin-DNA binding in peripheral leucocytes. The results of the phase I clinical and pharmacological study will indicate whether the phenomenon described here is of clinical relevance and may correlate with increased toxicity.

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(Received 23 September 1998; accepted 29 September 1998)