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QUARTERLY

Influence of G_2 arrest on the cytotoxicity of DNA topoisomerase inhibitors toward human carcinoma cells with different p53 status

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We here report the influence of the cell cycle abrogator UCN-01 on RKO human colon carcinoma cells differing in p53 status following exposure to two DNA damaging agents, the topoisomerase inhibitors etoposide and camptothecin. Cells were treated with the two drugs at the IC_{90} concentration for 24 h followed by post-incubation in drug-free medium. RKO cells expressing wild-type, functional p53 arrested the cell cycle progression in both the G_1 and G_2 phases of the cell cycle whereas the RKO/E6 cells, which lack functional p53, only arrested in the G_2 phase. Growth-arrested cells did not resume proliferation even after prolonged incubation in drug-free medium (up to 96 h). To evaluate the importance of the cell cycle arrest on cellular survival, a non-toxic dose of UCN-01 (100 nM) was added to the growth-arrested cells. The addition of UCN-01 was accompanied by mitotic entry as revealed by the appearance of condensed chromatin and the MPM-2 phosphoepitope, which is characteristic for mitotic cells. G_2 exit and mitotic transit was accompanied by a rapid activation of caspase-3 and apoptotic cell death. The influence of UCN-01 on the long-term cytotoxic effects of the two drugs was also determined. Unexpectedly, abrogation of

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Abbreviations: BCA, bicinchoninic acid; cis-Pt, cisplatin; CTP, camptothecin; PBS, phosphate-buffered saline; MTT, Thiazolyl blue; TBS, Tris-buffered saline.

the G_2 arrest had no influence on the overall cytotoxicity of either drug. In contrast, addition of UCN-01 to cisplatin-treated RKO and RKO/E6 cells greatly increased the cytotoxic effects of the alkylating agent. These results strongly suggest that even prolonged cell cycle arrest in the G_2 phase of the cell cycle is not necessarily coupled to efficient DNA repair and enhanced cellular survival as generally believed.

Exposure to DNA damaging agents leads to growth arrest in the G_1 and G_2 phases of the cell cycle thereby preventing the cells from replicating and separating the damaged DNA. The growth arrest is due to activation of different cell cycle checkpoints (surveillance proteins) which are activated by DNA damage or aberrant DNA replication. It is generally believed that the cell cycle arrest provides the cells with additional time to repair the potentially lethal DNA lesions, thereby rendering them more resistant to the cytotoxic effects of DNA damaging agents [1]. To overcome the cell cycle arrest induced by conventional cytotoxic anticancer drugs, new checkpoint abrogators, such as the staurosporine derivative UCN-01, have been developed. UCN-01 is able to block at least part of the stress signaling induced by DNA damaging agents, thereby triggering premature entry of the damaged cells into mitosis [2]. As expected, abrogation of the G_2 checkpoint is accompanied by increased cytotoxicity of many anticancer agents both *in vitro* and *in vivo* [3, 4]. UCN-01 is currently under clinical trials both as a single agent and in combination with other drugs as a cell cycle modulator [5].

The tumor suppressor protein p53 is a key regulator of stress-induced pathways which control DNA repair, cell cycle progression and cell death. In particular, p53 is required for G_1 but not for G_2 arrest although functional p53 appears to strengthen the G_2 arrest (for recent review see [6]). p53 is a transcription factor which can activate numerous genes through binding to specific DNA sequences in their promoter regions. The p53 protein is either absent or mutated in the majority of human cancers, including colon carcinomas. The influence of p53 on the cytotoxic effect of anticancer agents remains controversial [7, 8]. The use of genetic models where p53 function has been disrupted either by expression of the papillomavirus E6 protein [9–11] or where the p53 gene has been disrupted by homologous recombination [12] shows conflicting results since cells with non-functional p53 may become more sensitive, more resistant or have unchanged sensitivity depending on the agent and the cellular context. It has been proposed that the influence of UCN-01 may also be dependent on p53 status, since cells with non-functional p53 rely totally on the G_2 arrest (which can be modulated by UCN-01), whereas p53 proficient cells are also able to arrest damaged cells in G_1, which can not be overcome by UCN-01 [5]. In this study, we determined the influence of p53 function on the G_2 to M progression in RKO cells treated with two DNA damaging agents, the topoisomerase inhibitors camptothecin and etoposide — in the absence or presence of UCN-01. Our studies show that UCN-01 is able to provoke the G_2 to M transition in both etoposide- and camptothecin-treated cell irrespective of the p53 status. Unexpectedly, accelerated mitotic entry was not accompanied by enhanced cytotoxicity of the drug-treated cells strongly suggesting that cell cycle arrest and DNA repair are not necessarily coupled during the G_2 arrest as generally believed.

MATERIALS AND METHODS

Drugs, chemicals and antibodies. UCN-01 was kindly provided by Dr. E. Sausville (National Cancer Institute, Bethesda, MD, U.S.A.). Etoposide (VP-16), camptothecin, MTT (Thiazolyl blue), propidium iodide, RNase A were purchased from Sigma (St. Louis, MO, U.S.A.), caspase-3 activity kit and purified caspase-3 were from BioMol (Ham-

burg, Germany). Media, antibiotics and serum were from Gibco (Paisley, U.K.). Monoclonal anti-MPM-2 antibodies were from Dako (Carpinteria, CA, U.S.A.), monoclonal anti-cdc-2, polyclonal rabbit anti-p21, polyclonal rabbit anti-p53, polyclonal goat anti-actin were from Santa Cruz (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies were from Jackson ImmunoResearch Labs (West Grove, PA, U.S.A.). All other reagents were from local provider.

Cells. RKO/neo and RKO/E6 transfected cells were kindly provided by Dr. Joseph Bertino (Sloan-Kettering Institute, New York, NY, U.S.A.). Cells were maintained in MEM minimal essential medium supplemented with 10% foetal bovine serum and antibiotics (0.1 μg/ml streptomycin, 100 U/ml penicillin). The cells were grown in a monolayer culture at 37°C in a humidified 5% CO_2/95% air atmosphere. In these conditions the doubling time for both cell lines was 24 h. In some experiments, we also used RKO cells transfected with the empty vectro (RKO/neo). These cells behaved similarly to the parental RKO cells.

Cytotoxic activity. The cytotoxic activity of the drugs studied was determined by the MTT assay. Briefly, cells (1×10^4 per well) were allowed to attach in 24-well plates overnight before a 24-h exposure to drugs. Following two washes with warm growth medium, cells were incubated for an additional 96 h. The IC_{90} is defined as the inhibitory drug concentration causing 90% reduction of A_{540} absorbance *versus* that of control. In some experiments, 100 nM UCN-01 was added for 8 h following drug treatment.

Flow cytometry. Distribution of cells through the cell cycle was measured by flow cytometry using an EPICS Profile II Flow Cytometer (Coulter, Hialeah, FL, U.S.A.) equipped with an argon laser to give 488 nm light. The cells were fixed in 70% ethanol at –20°C, rehydrated in phosphate-buffered saline (PBS) and stained with PBS containing propidium iodide (20 μg/ml) and RNase A (100 μg/ml) for 30 min at room temperature. The percentage of cells in each phase of the cell cycle was calculated by MultiPlus software (Phoenix Flow Systems, San Diego, CA, U.S.A.). Biparametric flow cytometry analysis of MPM-2 and determination of the position of cells in the cell cycle was performed as described [13].

Caspase-3 activity assay. Drug-treated or control cells (1×10^6) were lysed in 50 μl lysing buffer (50 mM Hepes, pH 7.4, 0.1% Chaps, 5 mM dithiothreitol, 0.1 mM EDTA) for 5 min on ice, centrifuged at 20 000 × ***g*** for 10 min at 4°C and supernatants collected. Protein concentration was determined by the bicinchoninic acid (BCA) assay and supernatants were examined for caspase-3 activity using the caspase-3 kit (BioMol) according to the manufacturer's instructions. Fold increase in protease activity was determined by comparing drug-induced values and non-treated controls. Calibration curves were generated using purified caspase-3 (BioMol) and the activity was expressed in units/μg protein per h.

Western blotting. Cells were lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris/HCl, pH 8) containing protease inhibitor cocktail (Roche, Meylan, France) and phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 15 min on ice. Cell lysates were centrifuged at 20 000 × ***g*** for 10 min at 4°C and supernatants collected. Protein concentration in cell lysates was determined by the BCA assay. Equal amounts (50 μg per lane) were loaded in Laemmli buffer and separated by SDS/PAGE electrophoresis in 12% acrylamide gels and transferred onto PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). After transfer, membranes were blocked in 5% non-fat milk in TBS buffer (10 mM Tris/HCl, 150 mM NaCl, pH 8) and washed in TBST buffer (TBS buffer containing 0.05% Tween-20). Membranes were incubated with

primary antibodies diluted in TBST containing 0.5% bovine serum albumin at 1:100 (anti-p53, p21 and cdc2) or 1:1000 (anti-actin) for 1–3 h at room temperature. After three washes in TBST, membranes were incubated with secondary antibodies diluted at 1:40 000 in TBST for 1 h at room temperature. Results were revealed by the ECL kit (Amersham Pharmacia Biotech). Equal protein loading was verified by re-hybridization of the membranes and re-probing with anti-actin antibodies.

DNA fragmentation. DNA fragmentation in cells undergoing apoptosis was assayed essentially as described [14]. Following drug treatment, cells (2×10^6 per sample) were harvested by centrifugation, washed twice with ice-cold PBS, and lysed in 80 μl lysing buffer (10 mM Tris/HCl, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 7.4, and 0.5 mg/ml proteinase K) for 3 h at 50°C. Following lysis, the salt concentration (NaCl) was raised to 1 M and samples were centrifuged (30 min, 500 × ***g***). Supernatants were collected, DNA was precipitated with ethanol overnight at –20°C. The DNA pellet was dissolved in 20 μl 10 mM Tris/HCl, 15 mM NaCl, 1 mM EDTA, pH 7.4, RNA digested with RNase A for 0.5 h at 50°C (0.2 mg/ml final concentration) and samples separated by electrophoresis in 1.8% agarose gel with TBE as a running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) for 16 h at 1 V/cm. DNA was visualized by ethidium bromide staining (0.5 μg/ml, 1 h), destained overnight in re-distilled water, and photographed under UV illumination.

RESULTS

p53 function in RKO and RKO/E6 cells

The expression of p53 protein in RKO and RKO/E6 carcinoma cells was determined by Western blot analysis. The results show no detectable p53 expression in RKO/E6 cells (Fig. 1A, lane 1) and very low levels of p53 in untreated RKO cells (Fig. 1A, lane 2). In contrast, p53 protein levels are markedly enhanced in RKO cells after 24 h exposure to

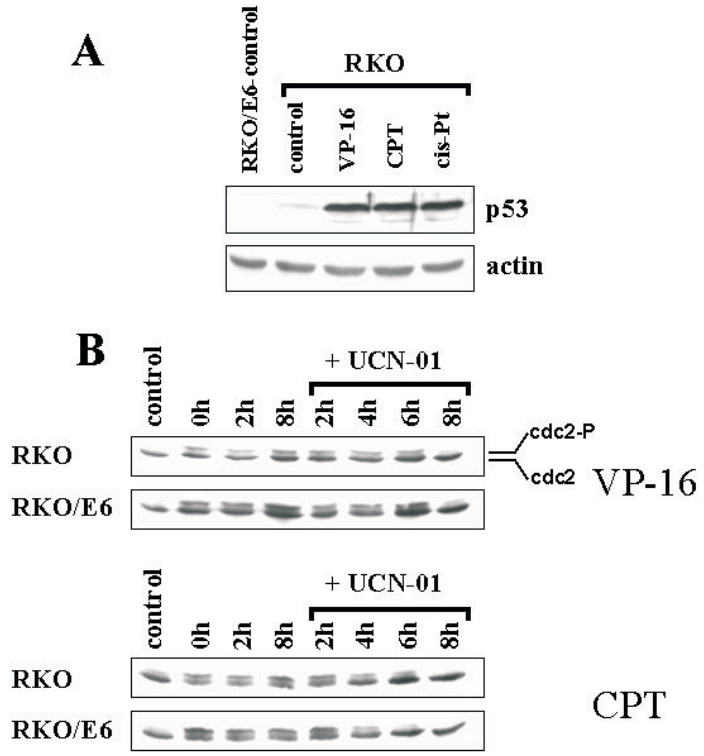

Figure 1. Panel A: Expression of p53 in non treated RKO and RKO/E6 cells or RKO cells treated with 5 μM VP-16, 50 nM camptothecin (CPT) or 5 μM cisplatin (cis- Pt) for 24 h as determined by Western blotting. **Panel B:** Cdc2 phosphorylation in RKO and RKO/ E6 cells treated with 5 μM VP-16 or 50 nM camptothecin for 24 h and post-incubated with or without 100 nM UCN-01, as determined by Western blotting.

VP-16, camptothecin or cisplatin (Fig. 1A, lanes 3–5). We also determined the induction of the cyclin dependent kinase inhibitor $p21^{cip1}$ which is transcriptionally regulated by p53 [15]. The results show that exposure of RKO cells to etoposide or camptothecin results in a more than 10-fold increase in p21 protein level as compared with untreated controls. In contrast, drug treatment had essentially no influence on p21 protein level in RKO/E6 cells (not shown). These results suggest that p53 is functional in the parental RKO cells but not in the RKO/E6 subline.

Influence of DNA topoisomerase inhibitors on cell cycle progression in RKO and RKO/E6 cells

The influence of etoposide and camptothecin on the cell cycle distribution was determined by flow cytometry after 24 h exposure followed by post-incubation in drug-free media. The DNA histograms show that parental RKO cells which express functional wild-type p53, are arrested in both the G_1 and G_2 phase of the cell cycle (Fig. 2A) whereas RKO/E6 cells, which lack functional p53, accumulate in the G2 phase of the cell cycle (Fig. 2B). Interestingly, the cell cycle arrest is remarkably stable, since none of the treated cells resume proliferation even after as long as 96 h post-incubation in drug-free media (not shown). Furthermore, it is worth noting that drug exposure is accompanied by formation of polyploid RKO cells as indicated by the presence of cells with an 8N content. This is most obvious for etoposide-treated cells, and to a lesser extent for camptothecin-treated cells (Fig. 2A). In contrast, no polyploid cells were observed for the RKO/E6 cells. To establish if

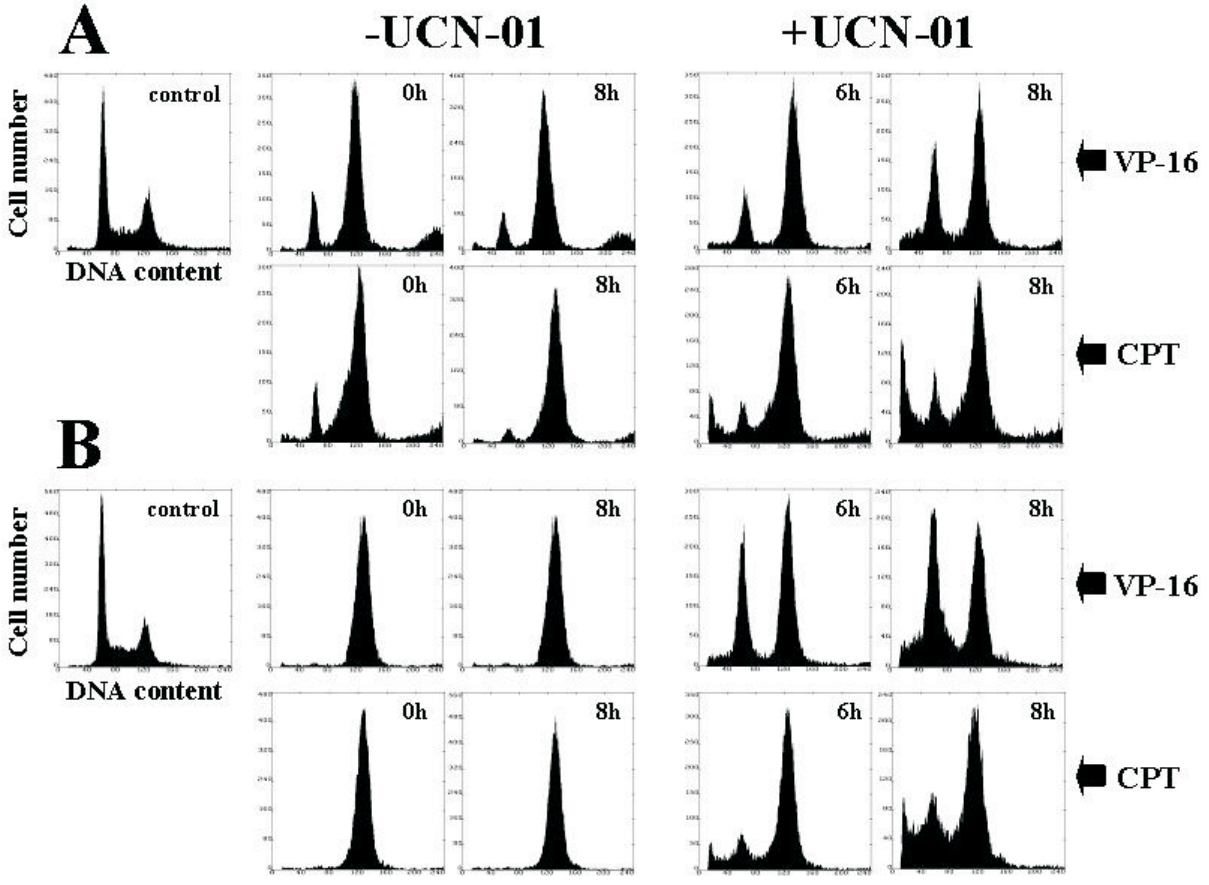

Figure 2. DNA histograms from RKO (panel A) and RKO/E6 (panel B).

Cells treated with 5 μM VP-16 or 50 nM camptothecin for 24 h and post-incubated with or without 100 nM UCN-01, as determined by flow cytometry. Abbreviations as in Fig. 1.

the cells were arrested in G_2 or in M, Western blot analysis was carried out for Cdc2 kinase, the major mitotic kinase. The results show that growth arrest is accompanied by the appearance of a new band with lower electrophoretic mobility in SDS/PAGE gels indicative of the phosphorylated inactive form of the kinase which is typical for cells in G_2 (Fig. 1B).

To determine the cellular consequences of prolonged G_2 arrest, a non-toxic dose (100 nM) of the cell cycle modulator UCN-01 was added to the growth-arrested cells for 8 h. The addition of UCN-01 results in rapid G2 exit and mitotic entry as revealed by the appearance of condensed chromatin and the MPM-2 phosphoepitope, which is characteristic for mitotic cells (not shown). G_2 exit is accompanied by gradual activation of Cdc2 kinase as shown by the conversion of the slow migrating phosphorylated inactive form into the faster migrating dephosphorylated active form of the kinase which is characteristic of mitotic cells (Fig. 1B). In the absence of UCN-01, no activation of Cdc2 kinase was observed throughout the 8 h postincubation period.

Induction of cell death

Incubation of drug-treated cells with UCN-01 was accompanied by the appearance of cells in G_1 and sub-G_1 (Fig. 2). This suggested that the G_2 to M transition may be accompanied by induction of apoptotic cell death. We therefore measured the activity of caspase-3, one of the major execution caspases. No measurable caspase-3 activity was observed in etoposide- or camptothecin-treated cells post-incubated in drug-free medium for up to 12 h (Fig. 3A). In contrast, post-incubation in the presence of UCN-01 is associated with a clear induction of caspase-3 activity. Interestingly, although the kinetics of caspase-3 activation is similar for the two drugs, the overall caspase-3 activity is about 10-fold higher in camptothecin-treated cells than in etoposide-treated cells suggesting differences in the cell death pathways induced

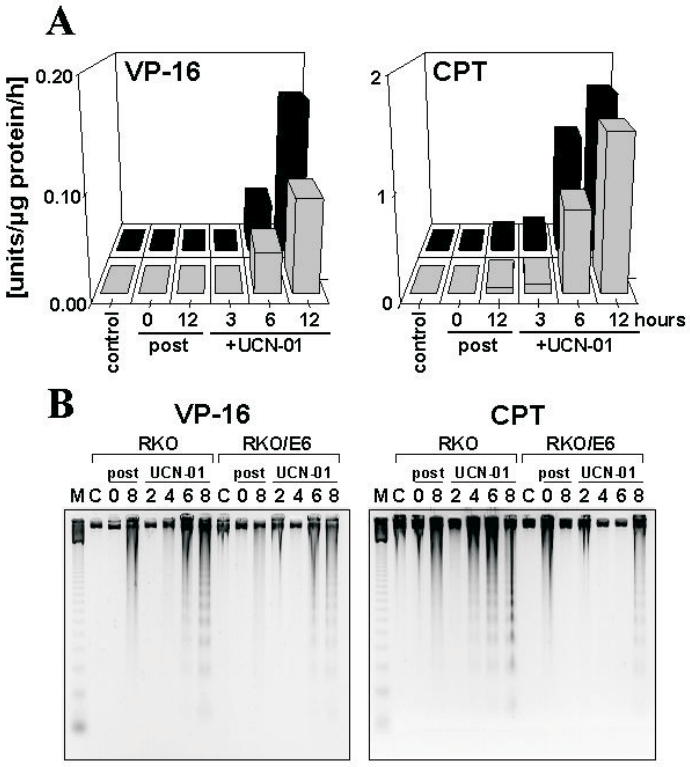

Figure 3. Panel A: Activation of caspase-3 in RKO (gray bars) and RKO/E6 (black bars) cells treated with 5 μM VP-16 or 50 nM camptothecin for 24 h and post-incubated with or without 100 nM UCN-01 for the time indicated. **Panel B:** Analysis of DNA fragmentation in RKO and RKO/E6 cells treated with 5 μM VP-16 or 50 nM camptothecin for 24 h and post-incubated with or without 100 nM UCN-01 for the time indicated. M, 123 bp DNA ladder; C, non-treated cells.

by the two drugs. We also determined if increased caspase-3 activity was accompanied by other markers of cell death such as DNA fragmentation. Increased DNA fragmentation is observed for drug-treated cells post-incubated with UCN-01, whereas no significant DNA fragmentation is observed in drug-treated cells which were post-incubated in drug-free medium for up to 12 h (Fig. 3B).

Influence of UCN-01 on the cytotoxicity of camptothecin and etoposide

Next, we wished to establish if the UCN-01 mediated caspase activation was accompanied by increased long-term cytotoxicity. Cells were treated with either drug for 24 h, post-incubated in the absence or presence of non-toxic doses of UCN-01 for 8 h followed by post-incubation in drug-free medium for an additional 4 days. Unexpectedly, in spite of the rapid induction of apoptotic cell death, UCN-01 had no influence on long-term cytotoxicity of either drug (Fig. 4). In contrast, postincubation with UCN-01 increased the cytotoxicity of cisplatin, a classical alkylating agent, 3-fold (not shown). Therefore, although UCN-01 successfully was able to overcome the G_2 arrest for all three DNA damaging agents, this was accompanied by increased long-term cytotoxicity for only one of them.

DISCUSSION

In the present study, we investigated the role of the tumor suppressor protein p53 in the G_2 to M progression induced by a well known checkpoint abrogator, a staurosporine derivative UCN-01. In particular, we were interested in long-term survival of G_2-arrested cells by sublethal doses of DNA topoisomerase inhibitors in which G_2 to M progression was induced by UCN-01. We used human colon carcinoma RKO cells expressing functional p53 protein and its subline with disrupted p53 function by expression of the E6 protein. We found that G_2 to M transition induced by UCN-01 occurred in both RKO cell lines independently of their p53 status. Surprisingly, this facilitated G_2 exit was not associated with enhanced long-term cytotoxic effect of camptothecin or etoposide.

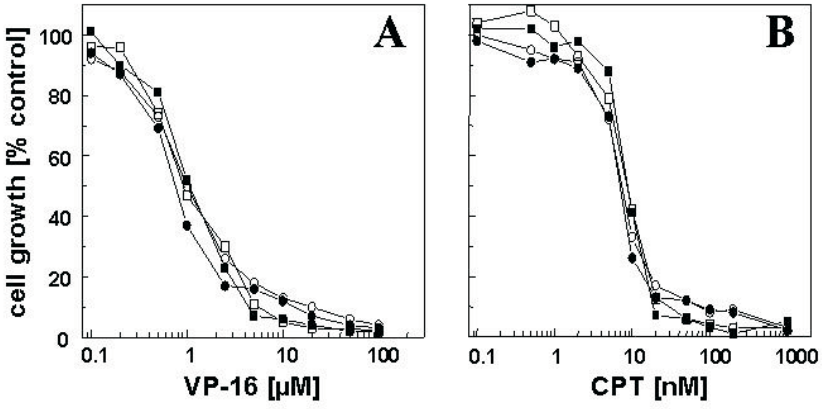

Figure 4. Loss of viability of RKO and RKO/E6 cells treated with VP-16 (A) and camptothecin (B) for 24 h and post-incubated with or without 100 nM UCN-01 for 8 h, as determined by the MTT assay after 96 h post-incubation in drug-free medium.

Symbols correspond to: (❐) RKO (■) RKO+UCN-01; (○) RKO/E6; (●) RKO/E6+UCN-01.

The role of p53 in G_2 arrest and in the G_2 to M transition in response to DNA damage has been somewhat controversial (for a recent review see [6]) and seems to be cell type dependent. Activation of p53 in myeloblasts from p53-null mice and in murine leukemia cells shortened G_2 arrest induced by ionizing radiation [16] and etoposide [13] and led to rapid induction of cell death and enhanced cytotoxicity. These and other results suggest that p53 may not only shorten G_2 arrest but also stimulate the induction of cell death by its pro-apoptotic activity during the G_2 to M transition. Consistent with this, it has been reported that abrogation of G_2 arrest induced by DNA alkylators such as mitomycin C and cisplatin by the checkpoint abrogators caffeine, pentoxiphylline or UCN-01 leads to enhanced cytotoxic effect [17] and synergistic antitumor activity *in vivo* [5]. However, for other drugs such as mitotic spindle poisons (taxol, paclitaxel, vincristine) combination with UCN-01 did not result in synergy [18].

There are also conflicting data concerning the role of p53 in the increased cytotoxic effect associated with combination with UCN-01 and DNA topoisomerase inhibitors. Several reports point to an enhanced activity of drug combination in cells with non-functional p53 [3, 19–21], whereas other show no influence of p53 status on cell sensitivity ([22], this study). Part of the confusion probably stems from the different treatment schedules used in those studies. In most of the cases, cells were treated simultaneously with both the DNA-damaging agent and UCN-01 [3, 19–22]. This treatment procedure leads to the situation where UCN-01 abrogates both the S-phase and G_2 checkpoints (see, e.g. [19–21]) and not the G_2 checkpoint alone (this study). As mentioned above, the mechanism of UCN-01 action is well defined only for the G_2 to M transition, and no detailed molecular mechanism concerning its role in S-phase checkpoint abrogation has been proposed. This is particularly surprising considering numerous reports where potentiation of drug cytotoxicity by UCN-01 through its abrogation of the S-phase checkpoint has been found (see, e.g. [19–21]).

The results of our study show that UCN-01 induces G_2 to M transition in RKO cells treated with all three drugs, i.e. camptothecin, etoposide as well as our reference compound, cisplatin, independent of p53 status. However, an increased cytotoxic activity of drug-UCN-01 combination was only observed for cisplatin. This result suggests that potentiation of cytotoxicity by abrogation of the G_2 checkpoint may be drug-dependent. This is in agreement with previous reports where pentoxiphylline, another DNA-damage checkpoint abrogator, failed to synergistically enhance the cytotoxic effect of taxol toward malignant glioma tumor cells [18]. Other studies have shown that UCN-01 combinations with paclitaxel and vincristine as well as topoisomerase II inhibitors, adriamycin and etoposide, do not result in synergy [3]. This apparent discrepancy between molecular events, i.e. the abrogation of drug-induced G_2 arrest by UCN-01 and the cytotoxic effect of drug-UCN-01 combination suggests several different possibilities. First, it is possible that in contrast to DNA alkylators, DNA damage induced in tumor cells at a lethal dose of camptothecin and etoposide is, at least in part, non-repairable by celullar defense mechanisms activated in G_2. In this case, irrespective of whether the drug-induced G_2 arrest is sustained by a DNA-damage checkpoint mechanism or is alleviated by UCN-01, this situation inevitably leads to cell death. In line with that, it has been demonstrated that UV-induced DNA repair, mainly nucleotide excision repair, is greatly reduced in RKO/E6 cells and dominant-mutant p53 transgenic RKO cells which also correlated with decreased clonogenic survival following UV-irradiation of cells with non-functional p53 [23]. Nucleotide excision repair is believed to be the main process by which DNA adducts induced by DNA alkylators such as cisplatin are removed from DNA, although

other pathways, such as mismatch repair, base excision repair and homologous DNA recombination, are also involved in DNA repair of such lesions [24]. Inhibition of DNA topoisomerases leads to the accumulation of single and double strand DNA breaks as well as undercondensed and entangled chromatids [25]. The majority of DNA breaks caused by topoisomerase inhibitors, especially those associated with the block of replication forks, are repaired by homologous recombination and non-homologous end-joining which are preferentially active in S/G_2 and G_1, respectively [26, 27].

Another possibility is that both DNA topoisomerase inhibitors studied have different mechanisms of action, one of which is activated during the S phase and the other operates only during the G_2 phase. The relative importance of the two mechanisms would probably depend on the drug concentration and consequently on the level of DNA damage induced by it. This possibility might be particularly applicable to camptothecin for which two cytotoxic mechanisms of action have been proposed, one of which is protectable by aphidicolin, i.e. depends on the ongoing DNA replication, and the other is not [28]. Finally, it is possible that cell death pathways induced by different drugs might differ and might depend or not on p53 status [29].

Together, our study shows that UCN-01 induces G_2 to M progression in tumor cells arrested in the G_2 phase of the cell cycle by sublethal doses of both camptothecin and etoposide, and the reference compound cisplatin, independently of p53 function. Unexpectedly, accelerated mitotic entry was not associated with enhanced long-term cytotoxic effect on cells treated with topoisomerase inhibitors. It follows that the ability of UCN-01 to overcome DNA damage-induced G_2 arrest was independent of both p53 function and the nature of the agent used to induce the DNA damage. However, the influence of G_2 abrogation on the long-term cytotoxicity was strongly agent-specific with no effect for both topoisomerase inhibitors studied and greatly enhanced cytotoxicity for cisplatin. These results strongly suggest that cell cycle arrest and DNA repair are not always tightly coupled during G_2 arrest as generally believed.

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