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Enhancement of the cytotoxicity of cisplatin by the cholecystokinin antagonist MK-329 in a human pancreatic cancer cell line

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Abstract. Cholecystokinin (CCK) is an important trophic hormone for the pancreas, and CCK receptors are present on pancreatic carcinoma cells. We sought to determine whether either CCK itself or an antagonist of CCK could modulate the sensitivity of the human pancreatic cell line MIA-PaCa2 to cisplatin (DDP). The IC_{50} for a 1-h exposure to DDP was 35.3 ± 3.2(SD) μ*M*. Exposure to CCK8 octapeptide at physiologic and supra-physiologic concentrations did not alter the sensitivity of MIA-PaCa2 cells to DDP. The CCK receptor antagonist MK-329 was directly cytotoxic to the MIA-PaCa2 cells on a constant exposure schedule with an IC_{50} of 9.5 ± 1.4 (SD) μ*M*. MK-329 enhanced the sensitivity of MIA-PaCa2 cells to DDP by a factor of 3.5, and the interaction between DDP and MK-329 was shown to be synergistic by median-effect analysis. At a level of 50% cell kill, the combination index was 0.58 ± 0.10. The ability of MK-329 to sensitize cells to DDP was schedule-dependent and required prolonged exposure to the antagonist following a 1-h exposure to DDP. MK-329 had no effect on the uptake of a radiolabeled analog of cisplatin ([^{3}H]-dichloro(ethylenediamine)platinum) and did not affect intracellular glutathione content. MK-329 had no effect on the cell-cycle-phase distribution of MIA-PaCa2 cells and did not alter the DDP-induced cell-cycle perturbations. The cytotoxic effect of MK-329 and its ability to interact synergistically with DDP could not be reversed by CCK. We conclude that MK-329 is directly cytotoxic to pancreatic carcinoma cells and can enhance their sensitivity to DDP by a mechanism not related to its ability to antagonize the CCK receptor.

Key words: Cisplatin – MK-329 – CCK – CCK antagonists – Synergy

Introduction

Carcinoma of the pancreas is the fifth most common cause of cancer death in the United States, carries one of the worst prognoses for all cancers [1], and poses one of the greatest diagnostic and therapeutic challenges in the field of oncology. At present, radical surgery in the form of the standard Whipple operation or total pancreatoduodenectomy offers the only hope for cure. In the small percentage of patients who present with tumors measuring less than 2 cm in diameter (T1a.N0.M0), these procedures may achieve 5-year survival rates as high as 37% [27]. The majority of patients, however, are not diagnosed at an early stage. Over 90% of patients will succumb to their disease within 5 years [22]. Chemotherapy for pancreatic cancer has been disappointing. Although radiotherapy plus 5-fluorouracil has been shown to increase survival marginally in resectable disease, there is no evidence that any single chemotherapeutic agent or combination alters the long-term survival of patients with advanced disease [4].

DDP has been used with considerable success in ovarian, testicular, and head and neck cancers and is playing an increasingly important role in combination modalities used for the treatment of gastrointestinal malignancies, particularly gastric cancer [17]. Gastrointestinal malignancies are frequently resistant to cytotoxic agents, usually requiring high-dose therapy that can result in significant sideeffects. Identification of ways to increase the sensitivity of tumor cells to chemotherapeutic agents without affecting systemic toxicity would constitute a major advancement in therapy.

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Abbreviations: DDP, cisplatin; DEP, *cis*-dichloro(ethylenediamine)-platinum(II); CCK, cholecystokinin; IC_{50}, concentration producing 50% cell kill in a clonogenic assay; CI, combination index; CI_{50}, combination index at 50% cell kill; GSH, glutathione; MBB, monobromobimane; PBS, phosphate-buffered saline

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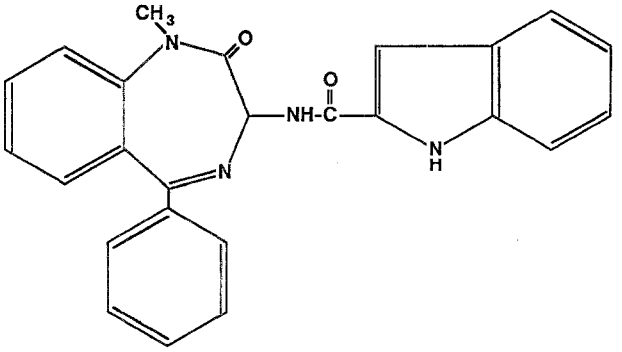

Fig. 1. Structure of MK-329

In recent studies it has been shown that the sensitivity of cancer cells to cytotoxic agents may be modulated by exposure of cells to growth factors or by activation of various growth-factor-receptor signal-transduction pathways. Epidermal growth factor, for instance, has been shown to sensitize ovarian cancer cells to DDP [10]. Somatostatin and luteinizing hormone-releasing factor have been shown to enhance the cytotoxicity of 5-fluorouracil in carcinogen-induced pancreatic cancer in hamsters [26]. There are also data to suggest that growth factor or hormone antagonists may enhance the cytotoxic effect of anticancer agents. Recently, tamoxifen has been shown to be synergistic with DDP in human malignant melanoma [21].

CCK is a major trophic hormone for pancreatic growth and development [12]. In recent years, potent and highly specific CCK receptor antagonists have been developed [29]. One such compound, MK-329 [7] (Fig. 1), is highly selective for the CCK-A receptor subtype, which constitutes the predominant receptor present in pancreatic tissue. We report herein that MK-329 interacts synergistically with DDP in the killing of human pancreatic cells but that this effect is independent of its blockade of the CCK receptors and is not mediated by effects on drug uptake or GSH content.

Materials and methods

Cell lines and tissue culture. All experiments were performed using the well-characterized human pancreatic carcinoma cell line MIA-PaCa2 [15], obtained from the American Type Culture Collection. Cells were maintained in culture using Dulbecco's modified Eagle's medium containing 4.5 g glucose/l and supplemented with 10% heat-inactivated fetal calf serum and 2 m*M* glutamine; all cultures were kept in a humidified incubator containing 5% CO_2 at 37° C. MIA-PaCa2 cells have a cloning efficiency of approximately 20% and form distinct colonies on plastic dishes.

Drugs. DDP was supplied by Bristol Myers-Squibb. MK-329 was obtained from Merck Sharp and Dohme Research Laboratories (West Point, Pa.) MK-329 was dissolved in dimethylsulfoxide (DMSO) prior to its use. The maximal concentration of DMSO added to each culture did not exceed 0.1%. CCK8 (fragment 26–33 amide, sulfated) was purchased from Sigma Chemical Co. (St. Louis, Mo.). CCK8 was dissolved in a minimal volume of DMSO prior to its final dilution with sterile PBS. [^{3}H]-DEP was synthesized as previously reported (11).

Clonogenic assay. Trypsinized cells were seeded at 400 cells/per 60-mm plastic tissue-culture dish (Corning Glass Works, Corning, N.Y.) in 5 ml medium; this seeding density yielded approximately 100 colonies after 7–8 days in culture. The cells were allowed to attach for 24 h, following which drugs were added to the cultures. After the appropriate period of exposure to drugs, the medium was removed and replaced with fresh non-drug-containing medium. Control plates received the appropriate drug vehicles. After 7–8 days of incubation, the medium was removed and the plates were washed with PBS, fixed with methanol, and stained with Giemsa; clusters containing more than 50 cells were counted as a colony. The total colony count was expressed as a percentage of the control-plate count. Colony-forming assays were performed using triplicate cultures for each data point, and each experiment was repeated three times. All data points represent the mean value ± SD for a minimum of three experiments, each being performed with triplicate cultures.

Median-effect analysis. Median-effect analysis was carried out to determine the nature of the interaction between DDP and MK-329. This was necessary because both drugs used as single agents exerted a cytotoxic effect, and it was important to distinguish an additive effect from a truly synergistic one [5]. Median-effect analysis [9] was performed on data obtained from clonogenic assays using combinations of the two drugs at different DDP and MK-329 concentrations, where the drug concentration ratio of each combination was kept constant at the ratio of the IC_{50} values. The effect of the combination was compared with that exerted by each drug alone, and the results were analyzed using the multiple-drug-effect analysis computer program described by Chou [8].

Schedule dependency of synergy. Preliminary studies indicated that to obtain synergy with a 1-h DDP exposure, a relatively long period of exposure to MK-329 was required. To establish the relationship between the interaction and the period of exposure to MK-329, a series of clonogenic assays using the drug combination were done in which cells were exposed to a fixed concentration of MK-329 (10 μ*M*) for either 0, 2, 4, 6, or 8 days. Exposure to DDP was carried out for 1 h and was started simultaneously with exposure to MK-329 as described for the previous experiments.

[^{3}H]-DEP accumulation. Cells were seeded at 500,000 cells/60-mm tissue-culture dish at 72 h before the start of the experiment so as to obtain approximately 2 million cells in each dish at the start of the uptake study. The cells were exposed to fresh medium containing 0, 10, or 20 μ*M* MK-329 (DMSO for control plates) and 5 μ*M* [^{3}H]-DEP at 5 μCi/ml. After 60 min of incubation at 37° C, the medium was aspirated and the dishes were washed three times with PBS (4° C). Cells were digested in 1 *N* NaOH, and the radioactivity was determined by liquid scintillation counting. The protein content was determined using the Bradford assay [6]. DEP uptake was expressed in picomoles per milligram of total protein. The experiment was repeated twice; the results presented represent the mean value ± SD for at least eight plates for each drug concentration.

GSH content. Cells were seeded at densities of 5,000, 10,000, and 15,000/cm^2 onto 60-mm culture dishes, were allowed to attach overnight, and were subsequently exposed to medium containing 20 μ*M* MK-329 (DMSO for control plates) for 0, 2, 4, or 6 days. After the appropriate period of drug exposure, cells were trypsinized, washed, aliquoted at 500,000 cells/Eppendorf tube, and pelleted for 1 min at 2,000 rpm. The medium was removed and GSH was derived by adding 50 μl MBB-TRIS (2 m*M* MBB, 20 m*M* TRIS-HCl, pH 8), mixing, and allowing the reaction to proceed for 15 min in a dark environment. An equal volume (60 μl) of 4 *M* sodium methane sulfonate was added to lyse cells and precipitate cellular proteins. The GSH-containing supernatant was assayed by high-performance liquid chromatography (HPLC) using 50-μl injection volumes and a Waters C18 μBondapak column and precolumn. Data collection and processing were carried

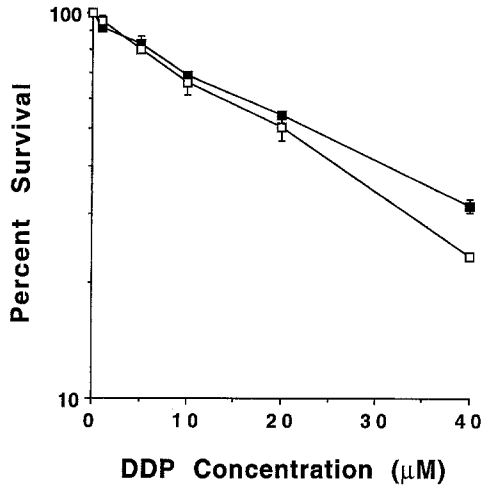

Fig. 2. Effect of CCK8 on the sensitivity of the MIA-PaCa2 cells to a 1-h exposure to DDP alone (■) and DDP plus 10 n*M* (□)

out on the Waters Maxima 820 system. The run time was 35 min with a retention time of 10 min. Each data point represents the mean value ± SD for six plates (duplicate plates at three different cell densities).

Cell-cycle analysis. The effects of DDP, MK-329, and the combination of the two drugs on cell-cycle-phase distribution was investigated using a 1-h exposure to 60 μ*M* DDP and continuous exposure to 20 μ*M* MK-329. Cells were synchronized by exposure to serum-free medium for 24 h, following which they were exposed to drugs and fixed in ethanol. Propidium iodide-stained cells were analyzed by flow cytometry using an Ortho Diagnostics Systems Cytofluorograph IIS equipped with a Data General 2151 data-analysis computer. A coherent Innova-90 argon laser was set to 50 mW at 488 nm excitation and red fluorescence above 610 nm was filtered by a glass long-pass filter. The data files were transferred to an IBM-compatible personal computer and cell-cycle analysis was performed using the Multicycle program.

Statistical analysis of data. All data points represent mean values ±SD. Data from clonogenic assays, median-effect analyses, and [^{3}H]-DEP uptake experiments were analyzed by the unpaired two-tailed Student's *t*-test. GSH content data were subjected to two-way analysis of variance (ANOVA). Statistical significance was set at the 95% level ($P < 0.05$).

Results

Drug sensitivity and median-effect analysis

The IC_{50} value obtained for a 1-h exposure of MIA-PaCa2 pancreatic carcinoma cells to DDP was 35.3 ± 3.2 μ*M*. This contrasts with the value of 2 μ*M* obtained for the human ovarian carcinoma cell line 2008 using a similar exposure schedule. Thus, the high IC_{50} value for the MIA-PaCa2 line reflects the usual clinical resistance of pancreatic carcinoma to DDP in vivo. MK-329 produced no cytotoxicity over a 1-h exposure period at concentrations of up to 60 μ*M*. However, on the continuous exposure schedule, the IC_{50} value was 9.5 ± 1.4 μ*M*.

Figure 2 shows that preexposure of cells to a physiologic concentration of CCK8 (10 n*M*) for 1 h had no demonstrable effect on the dose-response curve generated for DDP. Exposure to CCK8 on a constant exposure schedule,

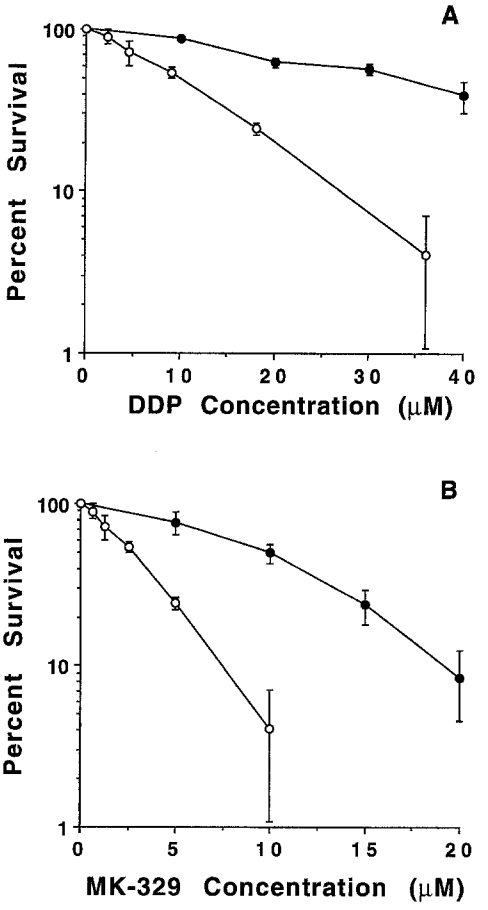

Fig. 3. **A** The effect of continuous exposure to MK-329 on the dose-response curve for a 1-h exposure to DDP alone (•) and DDP plus MK-329 (○). **B** The effect of a 1-h exposure to DDP on the dose-response curve for continuous exposure to MK-329 alone (•) and MK-329 plus DDP (○). Drugs were combined in a fixed molar ratio equivalent to the ratio of their IC_{50} values

even at supraphysiologic concentrations of up to 20 μ*M*, also failed to sensitize MIA-PaCa2 cells to DDP (results not shown). In contrast, Fig. 3A shows that continuous exposure to MK-329 resulted in a 3.5-fold reduction in the IC_{50} for a 1-h exposure to DDP at concentrations ranging from 35 to 10 μ*M*. Figure 3B shows the effect of the addition of a 1-h exposure to DDP on the dose-response curve generated for continuous exposure to MK-329. In a similar fashion, the addition of DDP led to a 3-fold reduction in the IC_{50} value for MK-329 at concentrations ranging from 10 to 3 μ*M*.

Although these figures show that there is an interaction between MK-329 and DDP, the nature of the interaction cannot be determined from such curves alone [5]. Therefore, median-effect analysis was undertaken to estimate the CI and, hence, determine the nature of the interaction. (A CI value of 1 indicates additivity, a value of >1 indicates antagonism, and a value of <1 indicates synergy). Figure 4 shows a plot of CI as a function of the extent of cell kill for a mutually exclusive interaction. It indicates that a high degree of synergy was present over the full range of the first log and a half of cell kill. The CI_{50} of 0.58 ± 0.10 (SD) was significantly less than 1 ($P < 0.05$).

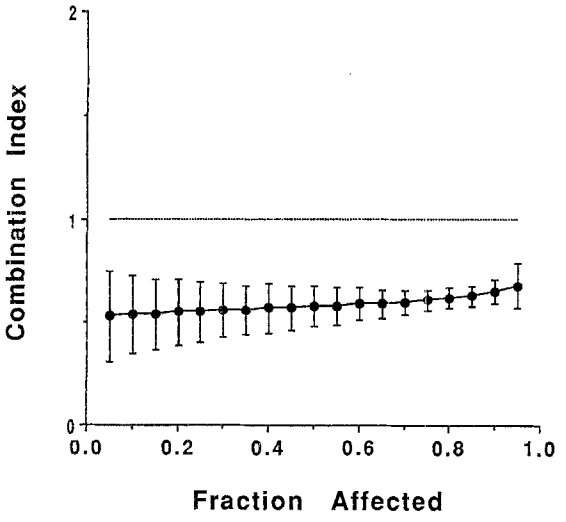

Fig. 4. Combination index plot for the interaction between DDP and MK-329 in MIA-PaCa2 cells. *Vertical bars*, SD

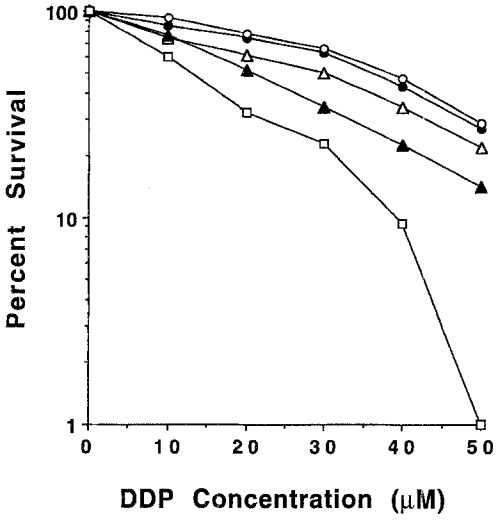

Fig. 5. Effect of the duration of exposure to 10 μm*M* MK-329 on the dose-response curve for a 1-h exposure to DDP alone (●) and to DDP plus MK-329 for 2 (○), 4 (△), 6 (▲), and 8 days (□). All SD values were <5%

Figure 5 shows the effect of the duration of MK-329 exposure on the ability of the CCK receptor antagonist to sensitize MIA-PaCa2 cells to DDP. When cells were treated with DDP for 1 h concurrently with MK-329 and MK-329 exposure was continued for only 2 additional days, there was no discernible sensitization to DDP. As the duration of post-DDP-treatment MK-329 exposure was increased from 4 to 8 days, the sensitization increased progressively as evidenced by the shift of the DDP dose-response curve to the left. The pretreatment of MIA-PaCa2 cells with MK-329 caused no sensitization to DDP in preliminary studies, and a median-effect analysis of such a schedule was not performed.

Although MK-329 is a highly specific inhibitor of the CCK-A receptor, it is not clear whether the cytotoxicity of the drug is mediated through its ability to inhibit the CCK

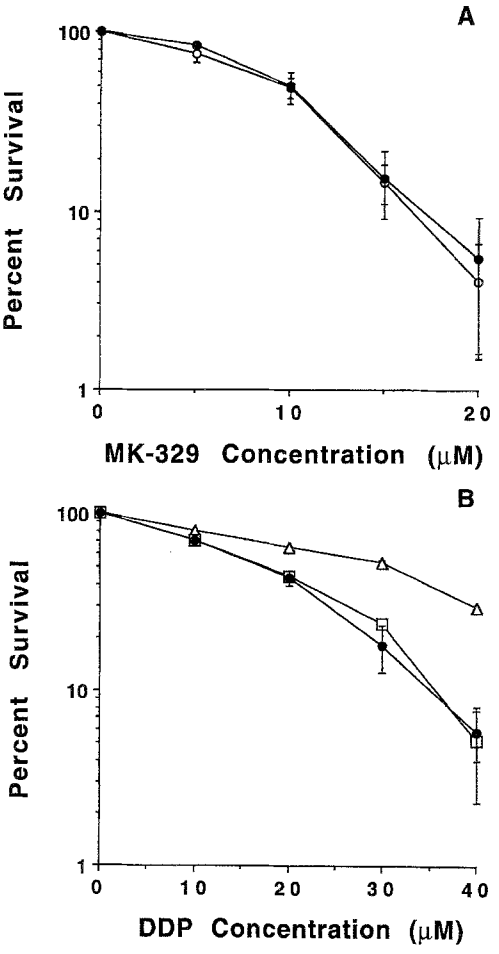

Fig. 6. **A** Effect of CCK8 on the cytotoxicity of MK-329 alone (○) and MK-329 plus CCK8 (●). **B** Effect of 10 μm*M* CCK8 on the interaction between DDP and MK-329. Δ, DDP alone; □, DDP plus 10 μm*M* MK-329; •, DDP plus 10 μm*M* MK-329 and 10 μm*M* CCK8

receptor. Figure 6A shows that for MIA-PaCa2 cells, the cytotoxic effects of continuous exposure to MK-329 could not be reversed or decreased by the addition of an equal concentration of CCK8 during exposure of cells to the CCK antagonist. Furthermore, the dose-response curve generated for DDP in the presence of a fixed concentration of MK-329 (10 μ*M*) was also not affected by the concomitant addition of 10 μ*M* CCK8 (Fig. 6B). Thus, the cytotoxicity of MK-329 and its ability to sensitize cells to DDP did not appear to be mediated through its inhibition of the CCK-A receptor.

[3H]-DEP accumulation

Modulation of drug uptake is one possible mechanism by which MK-329 may be enhancing cellular sensitivity to DDP. DEP is an analog of DDP that can be tritiated to a high level of specific activity, thus allowing cellular pharmacology studies. The uptake of [^{3}H]-DEP has been shown to reflect closely the uptake of native DDP [20]. The effect of MK-329 on the 1-h [^{3}H]-DEP uptake was examined in MIA-PaCa2 cells using 10 and 20 μ*M* MK-329. The DEP

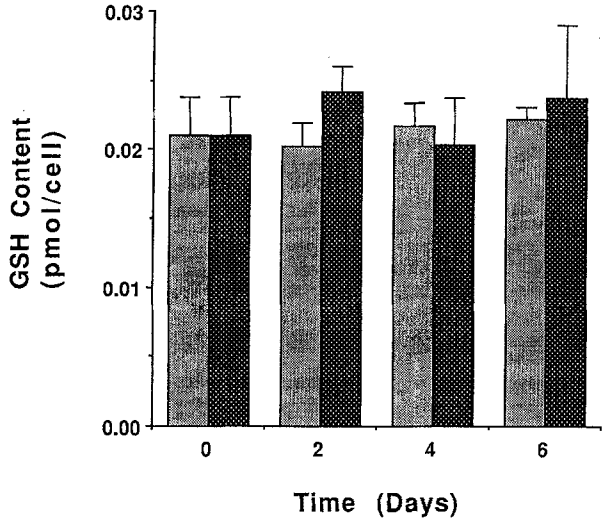

Fig. 7. Effect of 20 μ*M* MK-329 on the GSH content of MIA-PaCa2 cells over a 6-day period. *Lightly shaded bars*, controls; *heavily shaded bars*, MK-329; *vertical bars*, SD

uptake in the control cultures was 28.8 ± 1.2 pmol/mg protein. In comparison, uptake values for cultures containing 10 and 20 μ*M* MK-329 were 27.7 ± 1.2 and 30.2 ± 2.2 pmol/mg protein, respectively. Thus, there was no statistically significant effect of a 1-h exposure to MK-329 on [^{3}H]-DEP uptake. The effect of more prolonged exposures was not tested.

Effect of MK-329 on GSH content

GSH content has been reported to be a determinant of sensitivity to DDP. Figure 7 shows that exposure of cells to 20 μ*M* MK-329 for up to 6 days did not decrease their GSH content.

Cell-cycle effect

DDP causes arrest in the G_2 phase of the cell cycle, and the magnitude of this arrest is proportional to the extent of DNA adduct formation and repair. Figure 8 shows the effect of MK-329 on the cell-cycle-phase perturbation produced by DDP in a population of cells that were partially synchronized in the G_1 phase by 24 h of serum starvation. Control cells refed with serum-containing medium but not exposed to any drug resumed exponential growth as evidenced by an increase in the fraction of cells in the S and G_2 phases of the cell cycle at 24 h. MK-329 alone produced no perturbation of the cell-cycle-phase distribution. Cells treated with DDP demonstrated a marked accumulation in the S and G_2 phases of the cell cycle, followed by a gradual resolution of this perturbation by 96 h. When cells were treated with 20 μ*M* MK-329 along with 60 μ*M* DDP, there was a minor reduction in the extent of S and G_2 arrests at 24 h as well as a slight slowing of the resolution of the DDP-induced perturbation over the ensuing 72 h. These effects did not reach the level of statistical significance.

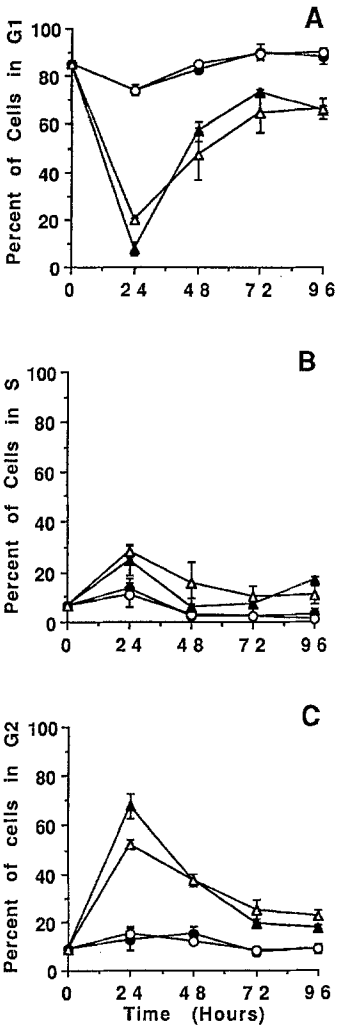

Fig. 8A–C. Effect of 20 μm*M* MK-329 and 60 μm*M* DDP on the cell-cycle-phase distribution in MIA-PaCa2 cells as determined by flow cytometric analysis of cells stained with propidium iodide; histograms show the percentage of cells in the different phases of the cycle. **A** G_1 phase. **B** S phase. **C** G_2 phase. •, Control; ○, MK-329; ▲, DDP; △, DDP plus MK-329

Discussion

Medical treatment of pancreatic cancer has been disappointing. This type of tumor is notoriously resistant to chemotherapy, including that containing DDP, and this is well reflected by the high IC_{50} value obtained for the MIA-PaCa2 cell line in vitro. Phase I/II studies with novel agents such as cytokines and monoclonal antibodies have not been any more therapeutically effective than palliative chemotherapy or radiotherapy [16]. Thus, the search for novel ways of making existing drugs more effective is gaining increasing importance in the hunt for new treatment strategies [4].

The concept that it might be possible to alter the sensitivity of pancreatic carcinoma cells using growth factors or growth-factor antagonists represents a new strategy. CCK is one of the most important hormones involved in pancreatic pathophysiology. It not only stimulates the

growth of the normal pancreas but also promotes pancreatic carcinogenesis and stimulates the growth of established tumors in vivo [23, 25]. CCK has also been shown to promote the growth of several human pancreatic carcinoma lines in serum-free medium, including the cell line used in our studies, MIA-PaCa2 [24]. These growth-promoting effects of CCK are mediated by the CCK-A receptor [28].

The discovery of CCK receptors in the gastrointestinal tract and the brain, together with the increasing realization of the importance of CCK in the pathophysiology of a multitude of gastrointestinal and neurologic disorders, has spurred the development of a diverse array of highly specific and very potent CCK antagonists [14]. CCK receptor antagonists can be classified into five categories: derivatives of cyclic nucleotides, peptides, amino acid derivatives, nonpeptide "peptoids" based on fragments of the CCK molecule, and benzodiazepine derivatives [29]. MK-329 belongs to the last category and is one of a series of synthetic compounds based on the structure of asperlicin, a naturally occurring benzodiazepine derived by fermentation from *Aspergillus alliceus* [14]. MK-329 is the most potent noncompetitive CCK-A receptor antagonist identified to date, showing a binding constant for the CCK-A receptor comparable with that of the native ligand CCK [7]. MK-329 is at least 100 times more potent than loxiglumide in antagonizing CCK-A receptors and has been shown to be effective by several routes of administration in modulating gastric emptying and gallbladder contraction [18]. MK-329 has been shown to inhibit the growth of subcutaneous human pancreatic carcinoma xenografts in nude mice [19], and we have now identified a direct cytotoxic potential in the pancreatic cell line MIA-PaCa2 in cell culture.

On the basis of our successful attempts at the modulation of DDP sensitivity with epidermal growth factor in the 2008 ovarian carcinoma cell line [10], we had initially sought to modify DDP sensitivity in the MIA-PaCa2 pancreatic cancer cell line using growth factors thought to be involved in pancreatic growth regulation. However, we failed to produce any enhancement of DDP cytotoxicity by CCK. Other growth factors such as gastrin, epidermal growth factor, bombesin, and the somatostatin analog octreotide also failed to enhance the cytotoxic effects of DDP (results not shown). We found, however, that MK-329 was cytotoxic to pancreatic carcinoma cells by itself when maintained at high concentrations over a long period and that it also sensitized these cells to DDP. The interaction between MK-329 and DDP was found to be truly synergistic by median-effect analysis.

We have investigated some of the mechanisms that may possibly be involved in this synergistic interaction. Although increased uptake of DDP has been implicated in some reported synergistic interactions [2], our results indicate that MK-329 did not increase the cellular uptake of DDP. Depletion of intracellular GSH has also been shown to enhance DDP toxicity [3]; however, MK-329 did not alter GSH levels in MIA-PaCa2 cells.

Another mechanism that may possibly underlie synergy with DDP is interference with the repair of DNA damage by premature induction of mitosis and prevention of the cell-cycle arrest in G_2 that is typically produced by DDP. This mechanism has been implicated in the synergy observed between DDP and methylxanthines such as caffeine [13]. We postulated that MK-329 may have a similar interaction with DDP; however, we failed to find any significant release of the G_2 block such as that observed with caffeine. Continuous exposure to 5 m*M* caffeine completely abolished the DDP-induced G_2 arrest in MIA-PaCa2 cells (results not shown).

Although the mechanism of the interaction between DDP and MK-329 remains unclear, it seems that CCK-A receptor blockade is probably not involved. The affinity of MK-329 for the CCK-A receptor has been reported to be in the nanomolar range, which is similar to that of CCK itself. Therefore, if its effect on sensitivity to DDP were being mediated via the CCK-A receptor, one might have expected the effect to occur at much lower MK-329 concentrations. Furthermore, we were incapable of reversing the cytotoxic effects of MK-329 applied either alone or in combination with DDP by using supraphysiologic concentrations of CCK.

It is also unlikely that the observed DDP/MK-329 interaction is mediated through the CCK-B receptor. In our preliminary clonogenic studies with MIA-PaCa2 cells, we failed to show any inhibition of cell growth by the CCK-B receptor antagonist L-365,260; this drug also failed to sensitize MIA-PaCa2 cells to DDP. L-365,260 [14], in contrast to MK-329, to which it is closely related, is a potent and highly specific CCK-B receptor antagonist and has a relatively weak affinity for the CCK-A receptor. Further indirect evidence against involvement of the CCK-A receptor in the DDP/MK-329 synergy is provided by the observation that MK-329 enhances the cytotoxicity of DDP against the human ovarian carcinoma cell line 2008, which does not express CCK receptors; in 2008 cells, MK-329 reduced the IC_{50} value for DDP by 45.3% $\pm$ 4.5% (R. Jamshidipour et al., unpublished data).

The observation that prolonged exposure is a prerequisite for synergy with DDP suggests that MK-329 may be interfering with a late-developing response to the DNA damage caused by DDP. The mechanism of action of the reported synergy and its possible therapeutic implications warrant further investigation.

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