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ORIGINAL ARTICLE

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A protein kinase C inhibitor induces phenotypic reversion of *ras*-transformed pancreatic cancer cells and cooperatively blocks tumor cell proliferation with an anti-*ras* peptide

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Abstract *Purpose*: We have previously found that the staurosporine derivative, CGP 41 251, that has a high specificity for inhibiting protein kinase C (PKC), selectively blocks oncogenic *ras*-p21-induced oocyte maturation and that PKC and *jun*-N-terminal kinase (JNK), with which oncogenic *ras*-p21 directly interacts, reciprocally require each other's activation. We sought to determine whether CGP 41 251 blocks proliferation of *ras*-transformed mammalian cells and whether it synergistically exerts this effect with a *ras*-p21 peptide (residues 96–110) that interferes with the interaction of *ras*-p21 with JNK. *Methods*: We incubated *ras*-transformed rat pancreatic cancer TUC-3 cells and their normal counterpart pancreatic acinar BMRPA1 cells with CGP 42 251 alone and in the presence of the *ras*-p21 96–110 peptide, both in pre- and post-monolayer phases and determined cell counts and morphology and, for TUC-3 cells, their ability to grow on soft agar. In the post-monolayer experiments, we also evaluated these parameters after withdrawal of these agents. *Results*: CGP 41 251, but not its inactive analogue, CGP 42 700, blocked pre-monolayer growth and reduced post-monolayer cell counts of both TUC-3 and BMRPA1 cells (IC_{50} 0.28 and 0.35 μM, respectively). After 2 weeks of treatment, all the remaining TUC-3 cells exhibited the untransformed phenotype. Withdrawal of CGP 41 251 resulted in almost complete regrowth of the normal BMRPA1 cells while the reverted TUC-3 cells grew much more slowly. These effects were greatly enhanced by the presence of the *ras*-p21 96–110 peptide. *Conclusions*: CGP 41 251 strongly blocks growth of *ras*-transformed pancreatic cancer cells by causing cell death and by induction of phenotypic reversion. The enhancement of this effect by the *ras*-p21 96–110 peptide indicated synergy between it and CGP 41 251, allowing it to block proliferation of the transformed cells selectively. These findings suggest the possibility of using these two agents in anticancer therapy.

Keywords Protein kinase C inhibitor · Pancreatic carcinoma cell lines · CGP 41 251 · ras-p21 96–110 peptide

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Introduction

Oncogenic *ras*-p21, but not its wild-type counterpart protein, induces mitogenic signaling pathways which result in transformation of mammalian cells [1]. In stage VI metaphase-arrested *Xenopus laevis* oocytes, microinjection of the oncogenic, but not wild-type, protein induces oocyte maturation [2]. Insulin also induces maturation via pathways that require activation of cellular *ras*-p21 [3].

We have found that a number of agents block oncogenic maturation induced by Ha-*ras*-p21 (containing the substitution of Val for Gly 12) but have little effect on insulin-induced maturation [4]. These agents include peptides corresponding to domains of p21 and its targets that have been found in molecular simulations to differ in conformation between oncogenic and activated normal proteins, such as the p21 35–47, 96–110 and 115–126 peptides [4], the SOS peptide 994–1004 [5], and the *raf* 97–110 peptide [6]. Since insulin-induced maturation depends on *ras*-p21 activation, we concluded that oncogenic and activated normal p21 proteins induce mitogenic signaling utilizing different pathways [4, 7].

Among the agents that selectively inhibit oncogenic p21-induced oocyte maturation is the staurosporine derivative, CGP 41 251, that, unlike the inactive, structurally related compound, CGP 42 700, selectively blocks the activity of protein kinase C (PKC) [8], which itself is a strong inducer of maturation [9]. Coinjection of CGP 41 251, but not CGP 42 700, with Val 12-p21 results in blockade of maturation while coinjection of the inactivating anti-p21 antibody Y13-259 with PKC results in no inhibition of maturation [9]. This finding suggests that PKC activation occurs downstream of *ras* on the oncogenic pathway. Since this derivative blocks PKC-induced oocyte maturation and oncogenic p21-induced maturation with identical dose-response curves, we concluded that inhibition of oncogenic p21 by CGP 41 251 is due to its blockade of PKC [9].

In immunoprecipitation [10] and in vitro binding experiments [10, 11], we have also found that Val 12-p21 binds with high affinity to the nuclear transcription protein, *jun* and its kinase, *jun*-N-terminal kinase (JNK), each of which, like PKC, induces oocyte maturation. This interaction is disrupted by the p21 96–110 peptide that strongly blocks Val 12-p21- but has no effect on insulin-induced oocyte maturation [10, 11]. Since the dose-response curve for peptide inhibition of Val 12-p21 binding to JNK can be superimposed on that for peptide inhibition of Val 12-p21-induced maturation [4], we concluded that the inhibitory effect of this peptide is due to its blockade of the Val 12-p21-JNK interaction. Since it does not inhibit insulin-induced maturation, which requires activation of normal cellular *ras*-p21, we further concluded that activated normal p21 does not interact directly with JNK/*jun* on its signal transduction pathway [4].

Further support for the requirement for JNK/*jun* activation of the oncogenic *ras*-p21 pathway derives from our finding that the enzyme glutathione-S-transferase (GST) is a highly selective inhibitor of the activation of *jun* by GST [12]. Coinjection of GST with Val 12-p21 into oocytes results in complete blockade of maturation while injection of GST into oocytes has no effect on the induction of maturation if the oocytes are subsequently incubated with insulin [13].

Since both JNK and PKC appear to be essential targets on the oncogenic but not activated normal p21 pathway, we further investigated the relationship between these two proteins on the oncogenic *ras* signal transduction pathway. Coinjection of CGP 41 251 with JNK results in complete inhibition of maturation, and, conversely, coinjection of GST with PKC also results in blockade of maturation [14]. These results suggest that PKC and JNK reciprocally activate one another and may interact in a synergistic manner [14].

CGP 41 251 has been found to induce cell death in two human transformed cell lines, T24, which are *ras*-transformed cells, and HL-60 human leukemic cells that are not known to harbor *ras* mutations [8], suggesting that PKC is also a critical element in *ras*-independent pathways. Though the IC_{50} values for this effect are somewhat higher for CGP 41 251 as compared with those for staurosporine itself, the IC_{50} values are significantly lower for CGP 41 251 in the same tumors explanted into athymic nude mice, indicating its potential efficacy as an antitumor agent [8]. These studies did not include an evaluation of the effects of CGP 41 251 on cell growth, i.e., whether it induces necrosis or induces phenotypic reversion of transformed cells. In addition, its effect on normal cell proliferation has not been systematically explored. Although it exerts an antiproliferative effect in bovine corneal epithelial cells, the ability of these cells to recover after withdrawal of the agent has not been determined.

In this study, we investigated the effect of CGP 41 251 on a *ras*-transformed pancreatic carcinoma cell line and a counterpart contact-inhibited untransformed pancreatic acinar cell line, both of which we have developed in our laboratory [15], with a view to establishing the effect of this agent on the *ras*-transformed phenotype and on the longer-term effects of this drug after it has been withdrawn from cell culture. Since PKC interacts in a positive manner with JNK [14], we further sought to determine whether CGP 41 251 and the Val 12-p21 peptide 96–110 cause synergistic/cooperative suppression of tumor growth in these cell lines.

Materials and methods

Cell lines

It was our objective to evaluate the effects of two agents, the anti-PKC compound, CGP 41 251 [8], and the *ras*-p21 96–110 peptide that blocks p21-JNK interactions [4, 10, 11] that have been found to block the mitogenic effects of oncogenic *ras*-p21 in *Xenopus laevis* oocytes, on *ras*-transformed mammalian cells.

We have developed two cell lines [15], one a normal-contact-inhibited line of rat pancreatic acinar cells, called BMRPA1.430 (BMRPA1) cells and the other a pancreatic acinar carcinoma line obtained by transfection of BMRPA1 cells with a plasmid containing an activated human K-*ras* oncogene (single base mutation at codon 12, valine substitution for the wild-type glycine in the ras protein, K-ras^{val12}; a kind gift from Dr. M. Perucho, CIBR, La Jolla, Calif.) and a neomycin resistance gene (J. Michl and L.Y. Bao, unpublished results). BMRPA1 cells have an epithelial cell phenotype, form acinar structures in culture, have no c-ki-*ras* nor p53 mutations, are unable to grow in anchorage-independent conditions and do not form tumors in Nu/Nu mice [15] (submitted for publication). In addition, they maintain their differentiated cell functions such as continued enzyme production and activation of

zymogen secretion by secretagogue. *Ras*-transformed BMRPA1 or TUC-3 cells were selected after transfection for their basis resistance to G418 and the overexpression of K-ras^{val12}. These cells no longer display an epithelial cell phenotype and acinar cell functions, but grow significantly more quickly than BMRPA1 cells and have a transformed spindle cell phenotype. In addition, we have found that these cells form colonies under anchorage-independent conditions in vitro and tumors in vivo in nude mice (Bradu et al., submitted for publication).

Protein kinase C inhibitor

The specific PKC inhibitor, CGP 41 251 [8], a staurosporine derivative, and the inactive counterpart staurosporine analogue, CGP 42 700 [10], used as a negative control in PKC microinjection experiments, were obtained as gifts from the Novartis (formerly, CIBA-Geigy) Corporation, Basel, Switzerland.

Peptides

Two peptides were synthesized for this study, one corresponding to the *ras*-p21 96–110 sequence (YREQIKRVKDSDDVP) and the other an unrelated control peptide from cytochrome P450, called X13 (MPFSTGKRIMLGE), both by solid phase methods and were obtained at >95% purity. A third modified peptide corresponding to the *ras*-p21 96–110 peptide with an aminoterminal fluorescein isothiocyanate (FITC) group attached was also synthesized (Peptide Technologies Corporation, Gaithersburg, Md.) and was also obtained at >95% purity.

Cell cultures

Both BMRPA1 and TUC-3 cells were grown in RPMI medium containing 10% fetal bovine serum (FBS) in an atmosphere containing 10% CO_2. Upon reaching approximately 80% confluence, cells were passaged, i.e., subjected to release by trypsin-EDTA (Gibco, Grand Island, NY), for continued growth or collected, adjusted to a predetermined cell concentration and, according to the experimental design, reseeded into 24-well tissue culture dishes or 25-cm^2 flasks.

In all experiments, cells were evaluated daily with respect to cell count (Particle Counter Z-1; Coulter Electronics, Luton, UK), trypan blue exclusion and morphology. Cell counts were also monitored using a Perkin-Elmer (Plainfield, N.J.) spectrophotometer model LS-5B. In some cases, crystal violet staining was used. Absorbance correlated linearly with cell count.

Two types of experiments, pre-monolayer and post-monolayer, were performed with these cells. In the pre-monolayer experiments, agents, i.e., staurosporine derivatives with or in the absence of a peptide, were incubated with the cells in growth phase. In these experiments, 5×10^5 cells were seeded into each well of 24-well tissue culture dishes containing 1 ml of culture medium. Cells were allowed to adhere for 24 h and the medium in sets of triplicate wells was then replaced with medium containing a staurosporine derivative with or without a peptide. The medium containing the agents(s) was replaced every 24 h.

In the post-monolayer experiments, cells were allowed to grow until the cell count reached that at which BMRPA1 cells form stable monolayers. Specific agents were then added to the medium and incubations performed for specific time periods (see legends to Fig. 4) after which the agent was withdrawn, i.e., the medium that was added no longer contained the agent. Cells were then incubated in the absence of agents until the growth curves reached a plateau.

Growth in soft agar

In experiments in which TUC-3 cells were incubated with CGP 41 251 alone or with *ras*-p21 96–110 peptide (see next paragraph), cells underwent phenotypic reversion as judged by their morphology. To confirm their untransformed phenotype, the ability of these cells to grow in soft agar [16] was determined. After growing to confluence the cells were trypsinized, and 1×10^4 were mixed with 0.37% Bactagar (Difco Laboratories, Irvine, Calif.) and then added to culture plates containing solidified 0.6% Bactagar medium. After the agar had solidified, the plates were incubated at 37°C for 2 weeks after which they were stained with a 0.001% solution of crystal violet, and colonies were counted. Simultaneous control experiments were performed in which untreated TUC-3 cells were subjected to the above procedure.

Incubations with peptides

To evaluate the efficiency of introducing the p21 96–110 peptide into TUC-3 cells, the FITC-labeled peptide was incubated with these cells at concentrations in the range 1–500 μg/ml. In these experiments, 5×10^5 cells were seeded into tissue culture dishes as described above. After 24 h, the medium was replaced by medium containing peptide. This process was repeated daily over a 12-day period. After various times, cells were observed for fluorescence using a Zeiss microscope equipped with epifluorescence and a fluorescein/rhodamine filter (Morrell Instrument Company, Melville, N.Y.). After 3 days, with peptide at 100 or 500 μg/ml, approximately 80% of the cells were found to contain punctate cytosolic fluorescence, a pattern that persisted for the entire time period.

Identical experiments were then performed with TUC-3 cells using unlabeled peptides at 100 or 500 μg/ml. These peptides were either p21 96–110 peptide or the X-13 control peptide. Each was incubated with TUC-3 cells either alone or in the presence of CGP 41 251 or CGP 42 700 at a concentration of 0.1 μg/ml. Cells were evaluated as described in the cell culture section above.

Results

Effects of CGP 41 251 on cell growth

Treatment of TUC-3 cells with different concentrations of CGP 41 251 over an 8-day period resulted in growth inhibition that was dose-dependent. As shown in Fig. 1 ((a typical time-course for inhibition), at a level of 1 μ*M* CGP 41 251, over 90% inhibition was achieved. Incubation of the cells with 1 μ*M* GCP 42 700, the inactive analogue of CGP 41 251, resulted in minimal inhibition,

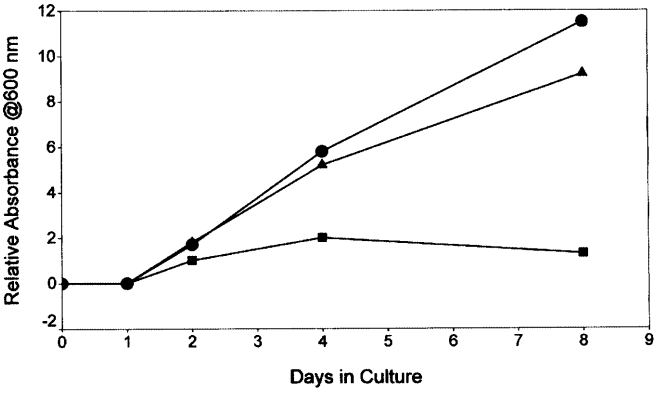


Fig. 1. Typical time course for inhibition of TUC-3 cell proliferation in the presence of 1 μ*M* CGP 41 251 (*filled squares*). CGP 42 700, the inactive staurosporine derivative caused virtually no inhibition (*filled triangles*) at the same concentration (*filled circles* results for sham-treated cells)

as also shown in Fig. 1. CGP 41 251 likewise induced a similar time-course for inhibition of growth of BMRPA1 cells with a similar dose-dependence (not shown).

Examination of cell growth inhibition by CGP 41 251 revealed two effects of this drug: rapid cell necrosis [8] (within 1 day of treatment) of both cell lines as found by staining cells with trypan blue at different time-points over the course of each experiment, and phenotypic reversion from an assessment of the cell morphology of viable cells over the same time course. Treatment of TUC-3 cells that had grown to confluence with 2 μM CGP 41 251 resulted in about a 25% reduction in the cell count due to necrosis, as judged by trypan blue exclusion. With no treatment or treatment with the GCP 42 700 negative control, TUC-3 cells appeared rounded and heaped up on one another as shown in Fig. 2B (upper right). After 8 days of treatment with 1 μM CGP 41 251, viable TUC-3 cells became non-heaped, enlarged and polygonal forming well-defined cell boundaries, with a different overall morphology from the parent BMRPA1 cells, as shown in Fig. 2D (lower right). These cells grew into a monolayer but were unable to grow in soft agar (no colonies formed after incubation in soft agar for 2 weeks) suggesting reversion to an untransformed phenotype, unlike untreated TUC-3 cells which formed colonies after 2 weeks of incubation.

These results can be compared with the results of treatment of untransformed BMRPA1.430 cells shown in Fig. 2C. Compared with the morphology of untreated cells (Fig. 2A) the cells were larger but the overall morphology remained similar. Treated TUC-3 cells (Fig. 2D) were significantly larger and had much larger nuclei than treated BMRPA1.430 cells (Fig. 2C).

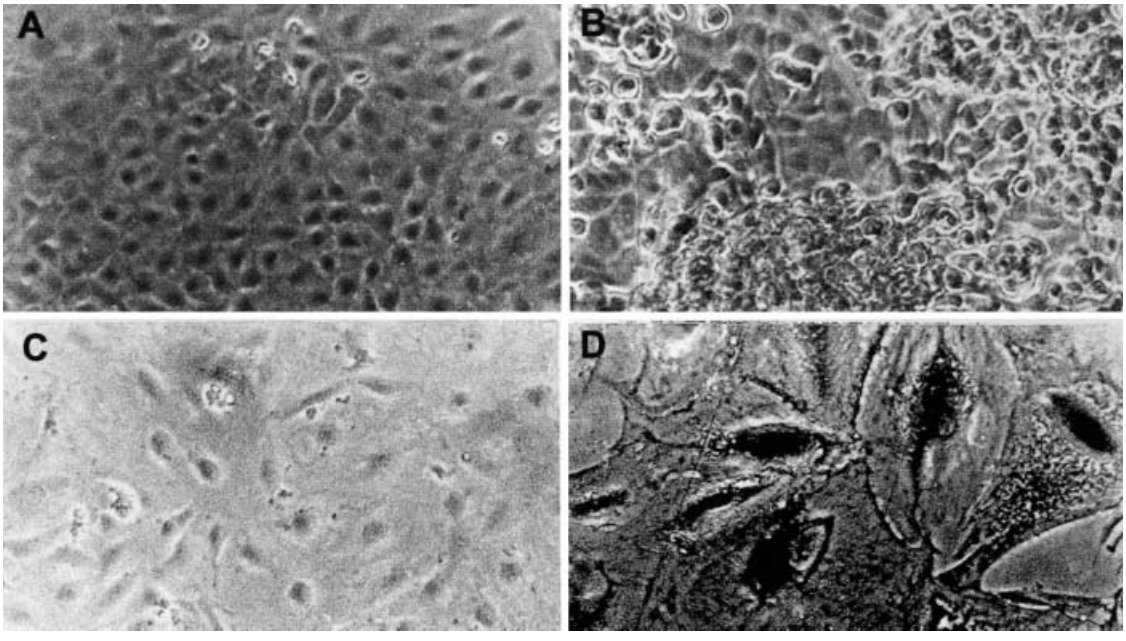
Fig. 2A–D. Photomicrographs of BMRPA1 cells in growth phase (**A**) and after treatment with 1 μM CGP 41 251 after 8 days (**C**) and TUC-3 cells prior to (**B**) and after (**D**) 8 days treatment with 1 μM CGP 41 251

Dose-dependence for growth inhibition

The dose-response curves for treatment of the two cell lines with CGP 41 251 were similar (Fig. 3). The IC_{50} values for this agent with TUC-3 and BMRPA1 cells were 0.28 μM and 0.35 μM, respectively. These values are similar to the IC_{50} values for the inhibition of growth of T24 cells (0.21 μM) and HL60 cells (0.30 μM). The in vitro IC_{50} value for inhibition of PKC by CGP 41 251 was 0.048 μM, which is about five times lower. However, since the intracellular concentrations of this derivative were not determined, it was not possible to compare these values directly. The IC_{50} values for inhibition of PKC-induced maturation of oocytes by CGP 41 251 were estimated to range from a maximum of 0.1 μM to a minimum of 0.008 μM, from an estimated 15-fold dilution of PKC in each injected oocyte [7].

Effects of withdrawal of CGP 41 251 on cell proliferation

Since arrest of cell growth occurred about equally for the two cell lines, normal and untransformed, we investigated the effect of drug withdrawal on the abilities of each cell type to proliferate. Cells were allowed to grow for several days to confluence at which time CGP 41 251 was added to the incubation medium daily. After a fixed time, addition of the agent was discontinued and the cells were observed until the growth curves reached a



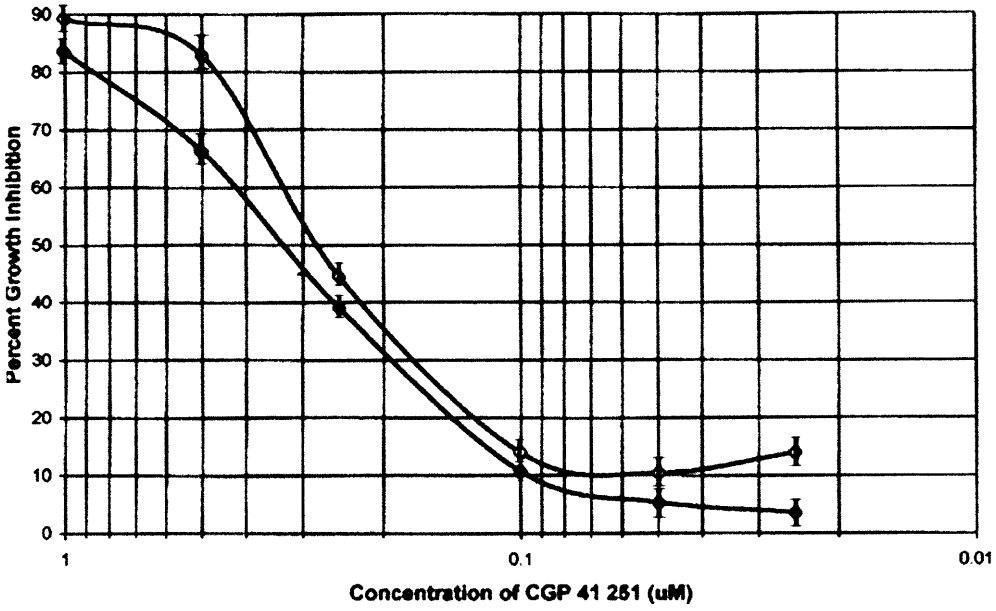


Fig. 3. Dose-response curves for growth inhibition of TUC-3 (*open circles*) and BMRPA1 (*filled circles*) cells. All data points represent the means of triplicate experiments; all standard deviations were $<2\%$ of the mean

plateau. Controls consisted of cells incubated with either CGP 41 251 or with its inactive homologue, CGP 42 700, continuously for the entire experimental period.

Substantial necrosis of untransformed BMRPA1 cells was observed. At the time of drug withdrawal, for the three doses of CGP 41 251 tested (0.5, 1.0 and 2.0 μM), the cell counts were reduced to 77%, 56% and 37% of the initial monolayer count (approximately 150,000 cells per plate), respectively. Importantly, at the end of the experiment these counts had increased to plateau levels that were 95%, 77% and 73%, respectively, of the monolayer count.

For untreated TUC-3 cells, proliferation resulted in cell counts that reached over sevenfold that for cells growing to cell counts at which BMRPA1 cells reached confluence. For treated cells, the cell counts at the time of drug withdrawal had increased about twofold at the lowest dose (0.5 μM) and remained close to the initial count at the two higher doses (1 and 2 μM). Inspection of the cells at different times during drug treatment revealed that cell death, phenotypic reversion and proliferation were occurring, suggesting that the relatively small changes in cell count resulted from a steady-state among these competing processes. Eventually, all viable cells exhibited an untransformed phenotype resulting in the observed plateau counts. These cells reached confluence after withdrawal of the agent at a concentration of 0.5 μM. Withdrawal of the agent at 1 and 2 μM resulted in plateau growth that was 80% and 43%, respectively, of the confluence value. Since all the cells displayed an untransformed phenotype, these different levels of plateau cell growth appeared to have resulted from different levels of necrosis vs phenotypic reversion. The former may have been dominant at the higher concentrations of CGP 41 251 while the latter may have prevailed at the lower doses.

Figure 4 summarizes the results of these experiments. In Fig. 4A, the plateau cell counts after drug withdrawal for TUC-3 cells at each of the three different concentrations of CGP 41 251 are plotted as percentages of the cell count for untreated cells grown over the same (3-week) period. For comparison, the results of continuous treatment of these cells with CGP 41 251 at each dose and with 1 μM CGP 42 700 (the inactive staurosporine derivative) are also shown. With continuous treatment with CGP 41 251, cell death, reflected as decreases in cell counts, increased with increasing drug concentration (Fig. 4). Cell counts decreased to 18% of the confluence count at 2 μM CGP 41 251 (compare the filled bar graph at 2 μM CGP 41 251 with the unshaded bar graph at 0.5 μM CGP 41 251 in Fig. 4A). As can also be seen in Fig. 4A, the growth of TUC-3 cells reached a maximum of about 50% of untreated cells (at the lowest concentration of agent, 0.5 μM) and, with the exception of the 1 μM CGP 41 251 point, increased by about twofold relative to the growth of the continuously treated cells. At 2 μM CGP 41 251, regrowth was minimal.

In contrast to the above results, for untransformed BMRPA1 cells (Fig. 4B) removal of the agent resulted in a major increase in cell count, approaching 100% of the original cell count (confluence) for untreated cells at 0.5 μM CGP 41 251 and about 75% of this cell count even at the two higher concentrations. Cell counts after withdrawal of CGP 41 251 at all concentrations reached control values for cells treated continuously with CGP 42 700 (Fig. 4B, Control). At 2 μM CGP 41 251, there was almost complete inhibition of cell growth with continuous drug treatment while, after drug removal, there was a 12-fold increase

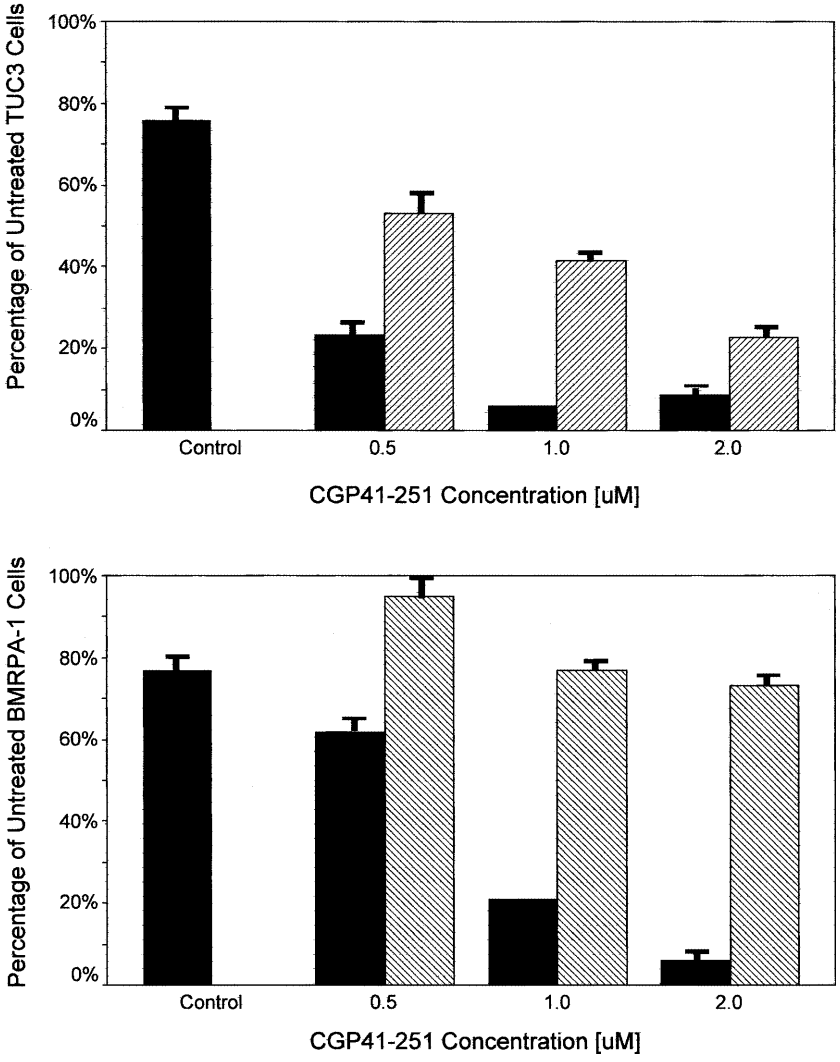


Fig. 4A, B. Effect of removal of CGP 41 251 on the growth of TUC-3 cells (**A**) and BMRPA1 cells (**B**) treated with this agent. All cell counts, measured both by absorbance and direct cell counting, are given as percentages of the cell counts for untreated cells. *Control* indicates the cell count for cells treated with 1 μ*M* CGP 42 700, the inactive staurosporine derivative (negative control). **A** TUC-3 cells were allowed to grow to confluence after which CGP 41 251 was either added (day 5) at the concentrations shown continuously for an additional 16 days (*filled bars*) or was administered for 9 days, after which it was withdrawn and the cells were observed for an additional 7 days (*hatched bars*). Plateau counts were achieved by 3 days after drug withdrawal. B The experimental conditions were the same as for the TUC-3 cells (**A**) except that the agent was added after 6 days and was continued for an additional 17 days (continuous treatment, *filled bars*). The cells were incubated with the agent for 6 days after which it was withdrawn, and the cells were observed for an additional 11 days. Plateau counts were achieved by 1 week after drug withdrawal. All experiments were performed in triplicate. The maximum standard error of the cell counts in both sets of experiments was 5%

in cell growth in relation to the CGP 42 700 negative control value.

Thus, while CGP 41 251 induced necrosis in both transformed and untransformed cells, removal of the drug allowed recovery of the growth of untransformed cells and complete phenotypic reversion of transformed cells that survived the treatment.

CGP 41 251 and *ras*-p21 96–110 peptide cooperatively block proliferation of transformed cells

We have previously found that PKC and JNK require reciprocal activation in induction of oocyte maturation [14]. Since the *ras*-p21 96–110 peptide inhibits Val 12-p21 interactions with JNK [4, 10, 11], we investigated the possibility of synergistic/cooperative interactions between the peptide and CGP 41 251 in blocking cell proliferation.

First we incubated the peptide by itself at a concentration of 100 μg/ml with TUC-3 cells. Since *ras*-transformed cells exhibit greatly enhanced pinocytotic activity [17], uptake of the peptide would be expected. As shown in Fig. 5, after 14 days of treatment, the cell count was reduced by approximately one-half while the control peptide, X13, had no effect on cell proliferation. Unlike the effect of CGP 41 251, however, this inhibition was not permanent and was overcome after another

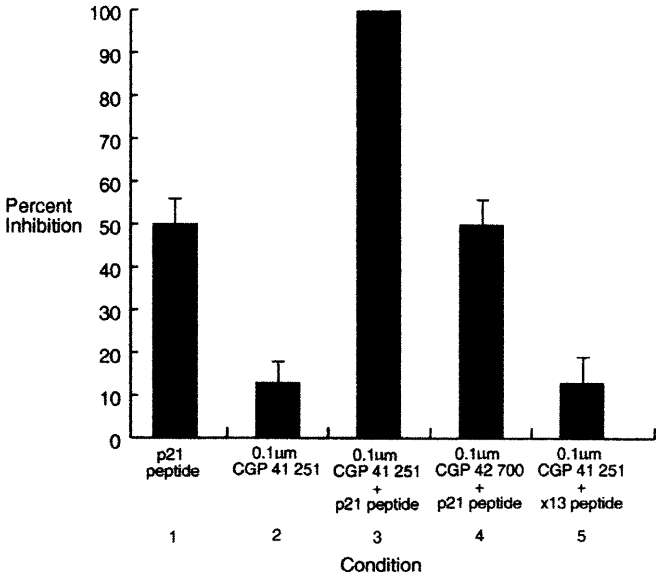


Fig. 5. Effect of 100 μg/ml of either *ras*-p21 96–110 peptide (third lane) or control X-13 peptide (second lane) on the growth of TUC-3 cells. Cell counts for untreated cells are given in *lane 1*

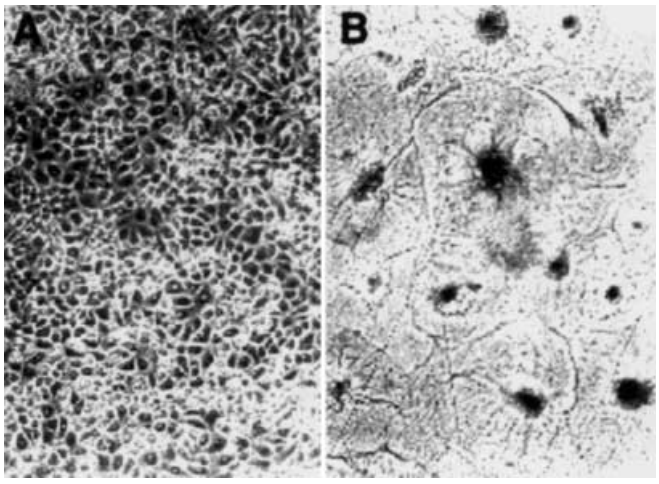


Fig. 6A, B. Effects of combined treatment with 0.1 μ*M* CGP 41 251 + *ras*-p21 96–110 peptide on the morphology of viable TUC-3 cells. **A** TUC-3 cell morphology just prior to treatment; **B** viable cells after 2 weeks of incubation with these agents

6 days. This effect was the same at 500 μg/ml of the peptide. This peptide did not inhibit the growth of BMRPA1 cells (not shown).

To test whether the peptide entered the cells, we incubated TUC-3 cells with the same peptide containing a fluorescent group (FITC) attached to its amino terminus. Over 80% of the cells were found to fluoresce, but all of these cells revealed a punctate pattern rather than a uniform distribution (not shown), suggesting that the peptide was contained in endocytic vesicles from which it was not released, possibly explaining the limited inhibition observed.

To explore cooperativity between the peptide and CGP 41 251, we coincubated these two agents with the peptide at 100 μg/ml and CGP 41 251 at 0.1 μ*M*, a concentration at which minimal (about 10%) inhibition was observed (Fig. 3). As shown in condition 3 of Fig. 5, 100% inhibition was achieved under these conditions. Since incubation of cells with the peptide alone (Fig. 5) caused 50% inhibition, there was about a twofold enhancement in the level of inhibition in the presence of CGP 41 251, present at a minimally effective concentration. Unlike the effect of the peptide alone, the inhibition observed from the combined treatment resulted in cessation of growth for the entire observation period of 21 days. at which time all the cells had reverted to the untransformed phenotype (Fig. 6). The morphology of the initially transformed cells prior to treatment with both agents (Fig. 6A) was the same as that of the cells shown in Fig. 2B. In contrast, viable cells after treatment for 21 days (Fig. 6B) appeared enlarged, had well-defined cell boundaries and were contact-inhibited, similar to the CGP 41 251-treated BMRPA1 cells shown in Fig. 2C.

As also shown in Fig. 6, the cooperative effect was specific for CGP 41 251 and the *ras*-p21 96–110 peptide. Incubation of TUC-3 cells with CGP 42 700 together with the *ras*-p21 peptide resulted in 50% inhibition, the baseline level for the peptide alone (Fig. 5, condition 4). Incubation of the cells with 0.1 μ*M* CGP 41 251 and 100 μg/ml of the inactive unrelated peptide, X13, resulted in the same level of inhibition induced by CGP 41 251 alone (Fig. 5, condition 5).

Identical results were achieved using 500 μg/ml of the peptide with 0.1 μ*M* CGP 41 251, indicating that maximal cooperativity was achieved at or below 100 μg/ml of the peptide. Importantly, incubation of BMRPA1 cells with the same concentrations of CGP 41 251 and peptide resulted in only minimal (about 10%) inhibition of cell growth (not shown).

Discussion

PKC as a target for CGP 41 251

Based on our previous results on the inhibition by CGP 41 251 of oncogenic *ras*-p21-induced oocyte maturation [7], we predicted that this agent should block proliferation of *ras*-transformed mammalian cells. We have now found that it induces growth inhibition of *ras*-transformed TUC-3 pancreatic cancer cells in a dose-related manner. This effect was specific since its inactive counterpart derivative, CGP 42 700, had little or no inhibitory activity. These results correlate with the abilities of these compounds to inhibit PKC [8], suggesting that PKC inhibition may be an important mechanism by which CGP 41 251 exerts its antiproliferative effects. On the other hand, the IC_{50} of CGP 41 251, about 0.35 μ*M*, similar to that found for T24 and HL-60 cells, i.e., 0.28 μ*M* [8], was about five times larger than the in vitro value for inhibition of PKC [8]. Although the intracellular concentration of this derivative is not known, and, as with oocytes, the agent may be diluted intracellularly [7], thus making the IC_{50}

value lower, the discrepancy in IC_{50} values suggests that CGP 41 251 may block other critical targets involved in cell proliferation, as discussed in the next section.

Two different responses to CGP 41 251

A major focus of this study was to define the nature of the growth inhibition. It is clear from Figs. 1 and 2D that at least events were induced to occur in transformed cells: cytotoxicity and phenotypic reversion. With respect to the former, cytotoxicity occurs in less than 24 h which suggests a nonapoptotic process, in agreement with the results of studies of the effects of CGP 41 251 on thymocytes [18]. In the latter studies, cell death occurred without DNA fragmentation in a ladder pattern. Cytotoxicity is also apparent when this agent is incubated with other normal cell lines, including BMRPA1 cells (e.g., Fig. 2) and corneal epithelial cells [8]. The second phenomenon, phenotypic reversion, occurs in a population of TUC-3 cells that form enlarged cells with enlarged nuclei which grow sluggishly and have no ability to grow on soft agar, unlike untreated TUC-3 cells. This effect was enhanced in the presence of the *ras*-p21 96–110 peptide (Fig. 6) as discussed below.

Importantly, withdrawal of CGP 41 251 from BMRPA1 cells resulted in significant regrowth of these cells as shown in Fig. 4B. Thus, while CGP 41 251 induced cytotoxicity in normally proliferating cells, this effect could be reversed simply by drug withdrawal.

Further significance of the twofold response to CGP 41 251

We have previously found that CGP 41 251 blocks microinjected oncogenic (Val 12-containing) ras-p21-induced oocyte maturation with the same dose-response curve as that for its inhibition of PKC-induced maturation [7]. On the other hand, it does not affect the ability of progesterone to induce maturation via a ras-independent pathway [7]. This result suggests that CGP 41 251 can block ras-p21-induced effects by blocking PKC without affecting general cell viability. This may represent the same pathway that results in phenotypic reversion of the pancreatic cancer cells, i.e., selective blockade of oncogenic ras-p21-induced activation of PKC in the oncogenic pathway. Since, however, this agent also induced significant levels of necrosis in the ras-transformed cells, blockade of other pathways may be involved that result in loss of cell survival.

Cooperativity between CGP 41 251 and *ras*-p21 peptide 96–110

Another major objective of this study was to determine whether CGP 41 251 and the *ras*-p21 96–110 peptide cooperate to block tumor cell growth. We found that PKC and JNK reciprocally require each other's presence in the induction of oocyte maturation [14]. Since the *ras*-p21 96–110 peptide blocks interaction of oncogenic Val 12-p21 protein with JNK and since CGP 41 251 blocks PKC, the presence of both of these agents might be expected to enhance each other's inhibitory effects. It can be seen from Fig. 5 that the presence of the peptide clearly enhanced the inhibitory effect of CGP 41 251 since this agent was present at a concentration causing only a minimal level of inhibition, suggesting cooperativity between these agents. Conversely, the presence of CGP 41 251 doubled the inhibition by the peptide and altered the nature of the inhibition such that cell growth became permanently arrested, also suggesting a positive interaction between the agents.

Cooperativity between these agents may prove advantageous in the treatment of *ras*-induced cancers. The *ras*-p21 96–110 peptide, either alone or together with CGP 41 251, had no effect on normal cell growth but caused ineffective doses of CGP 41 251 to become cytotoxic to *ras*-transformed cells. Therefore, the addition of this peptide to CGP 41 251 would significantly lower its effective dose to levels below any dose that would cytotoxic to normal cells, substantially increasing its efficacy. This finding suggests that these agents may be useful in the treatment of human malignancies.

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