Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2490–2494, March 1991
Biochemistry

A self-assembling protein kinase C inhibitor

(antineoplastic agent/synergism/hydrazone/lipophilic cation/prodrug)

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Communicated by Ronald Breslow, December 7, 1990

ABSTRACT **Previous studies have described a dicationic anticarcinoma agent that can chemically assemble *in situ* from monocationic phosphonium salts. The chemical combination of these monocationic precursors in the micromolar concentration range, occurring after their uptake by cells, was probably responsible for their synergistic inhibition of cell growth and for their selective cytotoxicity to Ehrlich ascites murine carcinoma cells relative to untransformed epithelial cells. Here, we report that the dicationic product that forms in this assembly reaction is an *in vitro* inhibitor of protein kinase C (PKC) α and β_1 isoforms, exhibiting IC_{50} values of 20.4 μM and 35 μM, respectively. The monocationic precursors proved to be much weaker inhibitors of PKC (IC_{50} values greater than 200 μM). When PKC is exposed to combinations of the two precursors, the enzymatic activity decreases steadily as a function of time. Using dose–response data and HPLC kinetic studies, we show that when the two precursor compounds are added as a combination to PKC under these conditions, the rate of formation of the inhibitory product follows the observed time course of decline in PKC activity under identical conditions. We discuss the possibility that antiproliferative effects against carcinoma cells of the preformed dication and of the combined monocationic precursors involve inhibition of PKC.**

One approach to combination chemotherapy of tumors involves "self-assembling antineoplastic agents"—i.e., direct covalent combination of less toxic prodrugs to form a more active drug *in situ* (1–3). When the prodrugs assemble into the active drug in or near cells, the prodrugs exhibit synergistic cytotoxic effects. If the chemical reaction between the prodrugs is more rapid in tumor cells than in normal cells, then the resulting enhancement of the tumor/normal concentration ratio of the assembled drug leads to an enhancement of the overall antineoplastic selectivity. This approach to prodrug combination design would be useful with a variety of macromolecular drug targets, including protein kinase C (PKC).

In recent years, investigators have focused on the significance of PKC as fundamental to the process by which extracellular growth factors propagate their growth signals from cell membrane to nucleus (28). During regulated cell growth, PKC responds to extracellular growth signals by phosphorylating specific target proteins on serine/threonine residues. Its kinase activity is stimulated by agonist-induced elevations in levels of Ca^{2+} and diacylglycerol (4), the latter being the product of agonist-induced breakdown of phosphatidylinositol (5). PKC activity is expressed by a family of at least seven structurally and functionally related isoenzymes (6) that exhibit slight differences in behavior (7) and whose distribution in the body is tissue specific (6). It is not known whether these differences engender specific sensitivities to growth signals or endow the isoenzymes with characteristic substrate specificities. PKC has also been identified as the receptor for the phorbol ester tumor promoters (4). This finding has further implicated this kinase as a key participant in the process of unregulated growth and, consequently, as a potential target for tumor chemotherapy (8, 9, 28).

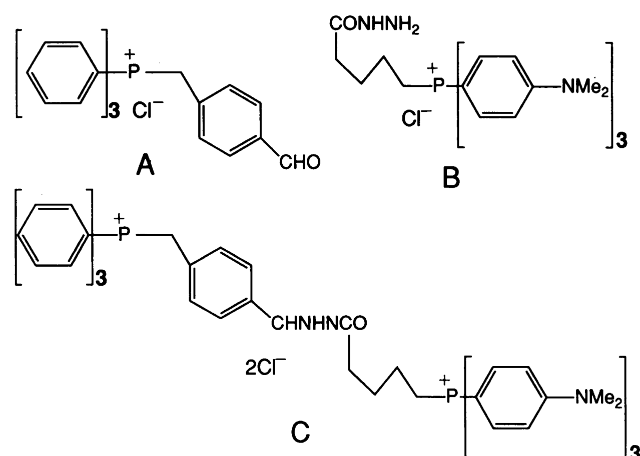

FIG. 1. Structural formulae of phosphonium salts **A**, **B**, and **C**.

Many of the most potent PKC inhibitors known are cationic and lipophilic or amphiphilic in nature (10). The lipophilic, dicationic, antineoplastic agent dequalinium chloride (11) is a much more potent inhibitor of PKC than are related monocationic molecules (12). These observations suggested to us that it would be possible to develop a combination of monocations that would synergistically inhibit PKC by combining to form a more potent dicationic inhibitor.

In this report, we describe synergistic PKC inhibition by combinations of monocations **A** and **B** due to self-assembly to form the dicationic hydrazone **C**, a much more potent inhibitor of PKC than the monocations (see Fig. 1). We also demonstrate a correlation between the observed PKC inhibition time course and the bimolecular reaction kinetics in combinations of **A** and **B**. To the best of our knowledge, the combination of **A**+**B** is the first example of an enzyme-inhibiting hydrazone that forms *in situ* from two less active precursors. The work also sheds light on the mechanism of carcinoma cell growth inhibition observed previously for combinations of **A** and **B** (3).

MATERIALS AND METHODS

Materials. DEAE-Sephacel was purchased from Sigma. Phosphatidylserine (PtdSer; synthetic dioleoyl compound)

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Abbreviations: PKC, protein kinase C; PtdSer, phosphatidylserine.
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was obtained from Avanti Polar Lipids. Polyclonal antisera were purchased from Seikagaku America. The epidermal growth factor receptor peptide was synthesized by J. Wideman (Columbia University). [γ-^{32}P]ATP (0.5–3 Ci/mmol; 1 Ci = 37 GBq) and Immobilon-NC 0.45-μm membranes were purchased from Millipore. Procedures for the preparation of **A** (13), **B** (14), and **C** (3) have been described elsewhere.

Methods. *Characterization of C.* Hydrazone **C** was characterized by fast atom bombardment positive-ion mass spectroscopy using a VG ZAB-VSE double-focusing high-resolution mass spectrometer. Mass calculated for $C_{55}H_{61}N_5P_2ClO^+$: 904.4040 Da. Mass found: 904.4001 Da. Other forms of characterization (NMR, IR) were reported previously (3).

Cell culture. Ehrlich–Lettre ascites (ELA) cells were plated onto 150-mm plates (Nunc) and cultured in Dulbecco's modified Eagle's medium containing 10% calf serum (Flow Laboratories). Cells were grown to confluence and harvested.

Isolation of PKC. The enzyme α-PKC was isolated from 8 g (wet weight) of ELA cells as follows. A whole cell lysate (45 ml) was prepared in 10 mM Tris·HCl, pH 7.5/5 mM 2-mercaptoethanol/0.25 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/soybean trypsin inhibitor (10 μg/ml)/0.1% Triton X-100. The lysate was centrifuged for 20 min at 9000 rpm in an SS-34 rotor (Sorvall) and the supernatant was chromatographed on a 1.7-ml DEAE-Sephacel column, as described previously (12). The eluate containing PKC activity exhibited a specific activity of 2.1 nmol of ^{32}P transferred per min per mg of protein. PKC was stored at −70°C in eluant buffer containing 10% (vol/vol) glycerol and 0.05% Triton X-100. Protein content was measured by using the Bio-Rad protein microassay with lysozyme as standard. From C3H/10T½ murine embryo fibroblasts genetically engineered to overproduce β_1-PKC (15, 16), the enzyme β_1-PKC was isolated by DEAE-Sephacel chromatography as described previously (12).

Western blotting analysis. Samples were subjected to electrophoresis (17) in SDS 8%/polyacrylamide gels at 40 V for 12 hr. Electroblotting onto nitrocellulose paper was carried out using a standard procedure (18). Immunoanalysis used three polyclonal antisera preparations (Seikagaku, Rockville, MD), each of which recognizes an epitope that is distinct for α, β, or γ isoforms of PKC. Detection of immune complexes was by use of an iodinated sheep anti-mouse IgG preparation (Amersham) followed by autoradiography at −70°C for 2 days.

Assay of PKC activity. PKC activity was taken as the difference in the rate of [^{32}P]phosphate transfer from [γ-^{32}P]-ATP to a synthetic peptide sequence of the epidermal growth factor receptor (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu) in the presence and absence of PtdSer at 83 μg/ml, as previously described (12). The reaction, conducted for 10 min at 30°C, was in 0.12 ml of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/0.5 mM $CaCl_2$ containing synthetic peptide substrate at 33 μg/ml, PtdSer at 83 μg/ml (or 10 μl of H_2O), PKC preparation at 167 μg/ml, and the test compound(s) added to the indicated concentration(s) as dimethyl sulfoxide stocks (final dimethyl sulfoxide concentration 3.3%, vol/vol). For β_1-PKC studies, PKC preparation was used at 16.7 μg/ml. The same conditions, with PtdSer present, were used for time-dependent PKC inhibition and HPLC kinetic studies (described below).

Control reaction mixtures typically contained 3.3% dimethyl sulfoxide instead of test compound(s). The reaction was initiated upon addition of 73 μM [γ-^{32}P]ATP (approximately 200 cpm/pmol). The reaction was quenched by transferring an aliquot to a 3 × 3 cm square of phosphocellulose paper, followed by repeated washings with water and measurement of ^{32}P content of each paper by scintillation counting. Each condition was tested in duplicate and the rates of kinase reaction were averaged. Duplicate values were routinely within 10% of each other.

Assay of time-dependent PKC inhibition. The time-dependent assembly of precursor compounds **A** and **B** into product compound **C** was carried out at 30°C and for increasing times of incubation (up to 60 min) with PKC in the presence of the kinase reaction components described above (including substrate and PtdSer), but without [γ-^{32}P]ATP. Components of the assembly medium were combined into glass tubes kept on ice; the assembly reaction was initiated by transfer of each tube to the 30°C water bath. Formation of compound **C** was allowed to occur for a given time period, after which [γ-^{32}P]ATP was added directly to initiate a 10-min kinase assay of the remaining PKC activity. All percent inhibition values were calculated relative to control phosphotransferase activities measured in parallel in the presence of PtdSer and Ca^{2+}, but without test compounds.

HPLC-based kinetics of C formation from A and B. The reaction conditions (for 200 μM **A** + 200 μM **B**) were almost identical with PKC assay conditions with PtdSer (see above), with radioactive ATP absent (20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/0.5 mM $CaCl_2$, containing synthetic peptide substrate at 33 μg/ml, PtdSer at 83 μg/ml, α-PKC preparation at 167 μg/ml, and 3.3% dimethyl sulfoxide, pH 7.5, 30°C). At periodic intervals, 40 μl of reaction mixture was combined with 360 μl of water containing 0.4 μg of rhodamine B (internal standard). This solution was immediately injected into a 0.46 × 25 cm (analytical) reverse-phase diphenyl silica column (Vydac 219 TP54). A gradient from pure aqueous 0.05% trifluoroacetic acid (TFA) to 70% aqueous TFA/30% acetonitrile (vol/vol) over 10 min and then to 40% aqueous TFA/60% acetonitrile over the next 15 min was used to separate **A**, **B**, **C**, and the internal standard rhodamine B (flow rate 2 ml/min, detection by UV absorption at 260 nm). The column was washed with 0.05% TFA in acetonitrile for 20 min between runs. The new peak formed from combinations of **A** and **B** coeluted with an authentic sample of **C**. As the time of incubation increased, the sizes of the **A** and **B** peaks decreased relative to the internal standard, while the sizes of the **C** peaks increased.

The standard curve used to quantify formation of **C** (y = quantity of **C** injected versus x = ratio of **C** to rhodamine B peak areas) was based on 15 points at concentrations ranging from 5 to 200 μM. The standard curve was developed by injecting 400 μl of an aqueous solution containing various concentrations of synthetic **C**, rhodamine B at 1 μg/ml, 2 mM Tris·HCl at pH 7.5, 1 mM $MgCl_2$, 50 μM $CaCl_2$, synthetic peptide substrate at 3.3 μg/ml, PtdSer at 8.3 μg/ml, α-PKC preparation at 16.7 μg/ml, and 0.33% dimethyl sulfoxide. These concentrations of Tris, $MgCl_2$, etc. were adjusted to match the concentrations in the diluted **A** + **B** reaction mixture immediately before injection in HPLC kinetic studies. The equation $y = ax$ was used to fit the data, and the resulting r value was 0.9894. For a given concentration of **C**, the peak size relative to the internal control was not affected significantly by the absence of PKC, peptide, and PtdSer.

When an equimolar mixture of **A** and **B** was injected into the HPLC without prereaction, a fraction was converted to **C** within the HPLC apparatus under our conditions. This fraction (14.6% ± 1.8%, mean ± SD, n = 7) was independent of concentration between 100 and 200 μM. It was also unaffected by PtdSer, peptide, and PKC. The actual amount of **C** formed from **A** and **B** during the incubation period (Z) was therefore derived from the amount of **C** observed in HPLC (Z_0) by using the equation $Z = 1.171Z_0 - 34.2$. The fraction converted to **C** after injection was significantly reduced by treatment of the mixture with excess aqueous butyraldehyde just before injection, presumably because hydrazone formation between butyraldehyde and **B** inhibited **C** formation from **A** and **B**.

RESULTS

The IC_{50} values for inhibition of PKC (isolated from ELA cells) are >200 μM for **A**, >500 μM for **B**, and 20.4 μM for **C**, (Fig. 2*A*). The respective IC_{50} values for β_1-PKC cloned in fibroblasts are >200 μM, >200 μM, and 36.0 μM (Fig. 2*B*). PKC isolated from ELA cells was found to consist of only the α isoform as determined by Western blot analysis (not shown). Immunochemical characterization of the β_1 isoform used for these studies has been reported (16). For both isoforms of PKC, dicationic hydrazone **C** was significantly more potent than components **A** and **B** from which **C** can be synthesized.

The IC_{50} values for **C** were calculated from an average of four determinations by using combination index methodology (19, 20). The corresponding *r* values exceeded 0.98. The calculated slope *(m)* values for **C** were 1.2 (α-PKC from ELA cells) and 1.94 (β_1-PKC). The *m* values for **C** calculated from individual experiments as opposed to aggregate data were 1.186 and 1.213 for the α-PKC isoform, compared with 1.657 and 2.28 for the β_1-PKC isoform. The IC_{50} values for **C** from individual experiments were 20.2 and 20.5 μM for the α isoform, as compared with 27.7 and 44.7 μM for the β_1 isoform.

The relative HPLC peak sizes for **C** (compared with the internal standard, rhodamine B) increased as a function of time, but relative peak sizes for **A** and **B** both decreased as a function of time of reaction in a combination of 200 μM **A** and 200 μM **B** under the conditions used for PKC formation (see *Materials and Methods* for conditions). Between 0 and 120 min of reaction, the average relative peak areas decreased 19.8% ± 5% for **A** and 18.5% ± 5.4% for **B** (two determinations). This corresponds to losses of 33.8 ± 8.5 μM for **A** and 31.6 ± 9.2 μM for **B**, when the reaction inside the HPLC column is taken into account (see *Materials and Methods*). The average increase in **C** concentration between 0 and 120 min was 38.1 ± 4.4 μM (error limits represent ranges, $n = 2$). The second-order rate constant k_2 for **C** formation from **A** and **B** under PKC assay conditions, based on the concentration of **C**, was 0.177 liter·mol^{-1}·sec^{-1} ($r = 0.934$). The time course for hydrazone formation calculated from this rate constant and the observed rate data are depicted in Fig. 3, together with a point representing hydrazone formation in the absence of PtdSer, α-PKC, and peptide substrate (8.3 μM **C** after 390 min).

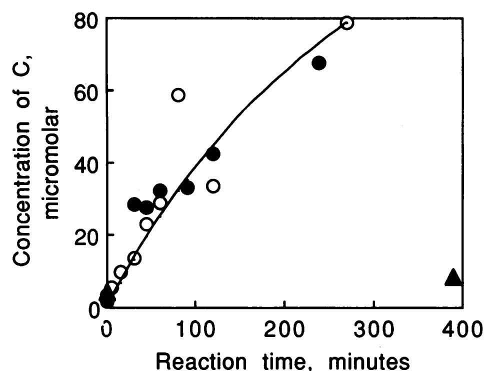

FIG. 3. Kinetics of the reaction between 200 μM **A** and 200 μM **B** under conditions used for PKC assays, as determined by HPLC. ○ and ●, Results from two independent experiments. The solid line is based on a second-order kinetic equation fit to these two sets of data points ($r = 0.934$). ▲, Result of a kinetic experiment carried out in the absence of PtdSer, PKC, and peptide substrate.

The PKC activity did not decrease significantly as a function of time during a 1-hr exposure to **A** (200 μM), **B** (200 μM), or **C** (25 or 50 μM) as single agents (Fig. 4*A*). In contrast, the enzyme activity dropped as a function of time during simultaneous exposure to equimolar **A** + **B** at 50, 100, or 200 μM each (Fig. 4*B*).

The time-dependent loss in α-PKC activity induced by 200 μM **A** + 200 μM **B** can be correlated with the rate of formation of **C** calculated from the HPLC data and the dose–response data for inhibition of α-PKC by **C**. These calculated data are represented by the solid line in Fig. 4*B*. The percentage of control PKC activity at time $t = 0$ is 48.3% rather than 100% for 200 μM **A** + 200 μM **B** (see Fig. 4*B*). This can be explained by PKC-inhibitory activity caused by **A** and **B** (see Fig. 2*A*) and by the small amount of **C** formed during the 10-min PKC assay. The time-dependent loss of PKC activity during exposure to 200 μM **A** + 200 μM **B** was calculated by applying the median effect equation (19, 20) to the average concentrations of **C** measured by HPLC after 10, 25, 40, 55, and 75 min of reaction, using the following equation: $P(t) = 48.3 \times \{1 - [1/[C_m/C(t)]^m]\}$. Here $C(t)$ is the concentration of hydrazone **C** formed at time *t* after the reaction between **A** and **B** begins, $P(t)$ is the PKC activity as a percentage of control, C_m is the IC_{50} for inhibition of α-PKC by hydrazone

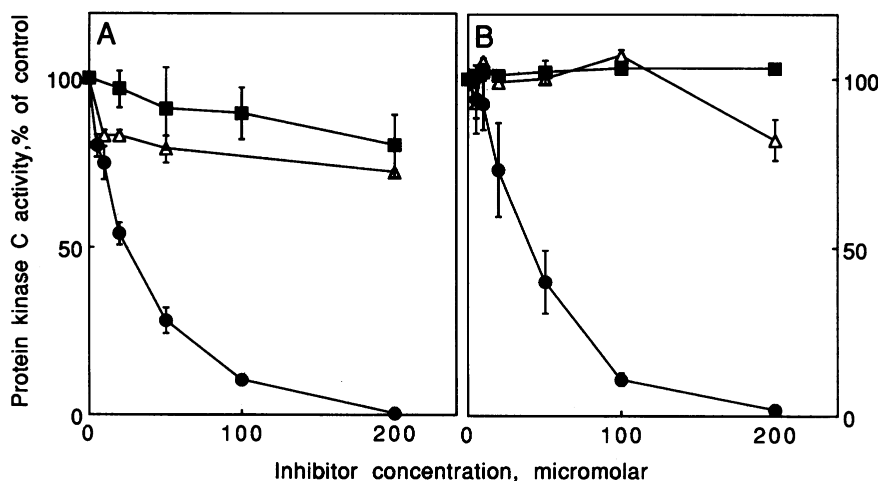

FIG. 2. Inhibition by phosphonium salts of PKC. Inhibition studies were carried out at 30°C, pH 7.4, as described in the text. ■, Inhibition by **A**; △, inhibition by **B**; ●, inhibition by **C**. The error bars represent the range. No error bar is shown when the width of the symbol exceeds the range. (*A*) Inhibition of α-PKC isolated from ELA murine carcinoma cells. Mean of two determinations for **A**, six for **B**, and four for **C**. (*B*) Inhibition of β_1-PKC isolated from murine fibroblasts transfected with a gene encoding β_1-PKC. Average of two determinations for **A** and **B** and four for **C**.

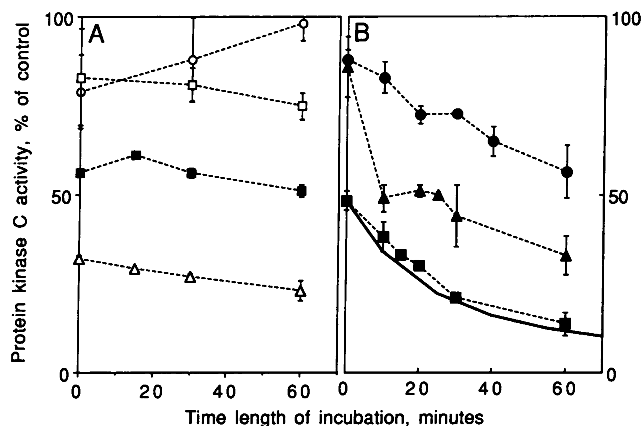

FIG. 4. Time dependence of inhibition of α-PKC (isolated from ELA cells). The times represented on the abscissa do not include a 10-min period required for the PKC assay itself. All points represent means of two observations. Error bars represent the range. No error bar is shown when the width of the plot symbol exceeds the range. (*A*) Inhibition by **A** alone, **B** alone, and **C** alone. ○, 200 μM **A**; □, 200 μM **B**; ■, 25 μM **C**; △, 50 μM **C**. (*B*) Inhibition by equimolar combinations of **A** and **B**. ●, 50 μM **A** + 50 μM **B**; ▲, 100 μM **A** + 100 μM **B**; ■, 200 μM **A** + 200 μM **B**. The solid line represents predicted α-PKC activity (relative to the control) after exposure to 200 μM **A** + 200 μM **B**. This line was calculated from HPLC kinetic data points (Fig. 3, PtdSer and peptides present) and the PKC inhibition dose response for **C** as detailed in *Results*.

C (20.4 μM), and *m* is the slope parameter (1.2). The results of this calculation are depicted as a solid line in Fig. 4*B*.

DISCUSSION

Combinations of aldehydes and hydrazine derivatives can exhibit cytotoxic synergism due to the formation of more cytotoxic hydrazones *in situ* (1–3). As reported by some of us in a previous publication (3), the combination of aldehyde **A** and hydrazine derivative **B** (Fig. 1) is particularly interesting in that it exhibits greater synergism against ELA murine carcinoma cells than against untransformed CV-1 monkey kidney epithelial cells. The combination indexes (19, 20) for combinations of **A** and **B** are less than 0.7 against ELA, indicating synergism [80% inhibition level, mutually exclusive equation, 1.31:1 and 5.3:1 ratios of **A** to **B** (3)]. In contrast, the combination indexes for **A** and **B** against CV-1 untransformed cells are greater than 1.1, indicating antagonism [80% inhibition level, 2.61:1 and 6.49:1 ratios of **A** to **B** (3)]. Both **A** and **B** resemble tetraphenylphosphonium, which accumulates selectively in ELA and other carcinoma cells relative to CV-1 cells due to higher transmembrane potentials (3, 14, 21, 22). The carcinoma-selective synergism observed for combinations of **A** and **B** is probably due to selective accumulation of both **A** and **B** inside the carcinoma cells, followed by selective condensation to form the more cytotoxic hydrazone (**C**) inside the tumor cells (3). This carcinoma-selective synergism is particularly interesting in terms of a rational design of drug combinations with enhanced therapeutic indexes (1, 3, 23).

We have carried out studies of the effects of **C** and **A** + **B** on PKC to help clarify the mechanism of action of **C** and the mechanism of synergism of **A** and **B**. We have found that combinations of **A** and **B** induce a striking time-dependent decrease in PKC activity that can best be explained in terms of assembly of the more potent PKC inhibitor **C** *in situ*. In addition, **C** is interesting as a PKC inhibitor in its own right because of the unique aspects of its chemical structure and possible selectivity in the inhibition of different isoforms of PKC.

The hydrazone **C** inhibits PKC with a potency (IC_{50} = 20.4 μM) similar to that of dequalinium chloride (12) and alkylglycerol derivatives (24). Hydrazone **C** is a significantly more potent inhibitor of PKC than either aldehyde **A** or acylhydrazide **B**, from which it is formed (IC_{50} values >200 and >500 μM, respectively). The hydrazone **C** is somewhat less potent in its inhibition of β_1 isoform as compared with the α isoform isolated from ELA cells (IC_{50} = 36.0 versus 20.4 μM, respectively). **C** exhibits a steeper inhibition curve (m = 1.9 versus 1.2, respectively), suggesting a more cooperative interaction of **C** with the β_1 isoform. These data suggest that the mechanism of inhibition by **C** may differ significantly for the two isoforms. However, the differences are modest, and verification of isoform selectivity must await further experiments.

A combination of aldehyde **A** and hydrazide **B** exhibits synergistic inhibition of α-PKC. For example, after 1 hr, a combination of 50 μM **A** and 50 μM **B** inhibits α-PKC by 40%, whereas 200 μM **A** alone and 200 μM **B** alone each inhibit the enzyme by less than 25%. The combination index under these conditions is therefore less than 0.57, where a combination index value less than 1.0 indicates synergism (19, 20). The inequality holds for both mutually exclusive and mutually nonexclusive equations (19, 20). The degree of enzyme inhibition by combinations of **A** and **B** increases steadily with time over 1 hr, in contrast to **A** alone, **B** alone, and **C** alone (Fig. 4). Preliminary results suggest that inhibition of β_1-PKC by combinations of **A** and **B** is also synergistic (unpublished observations). The synergistic inhibition of PKC by combinations of **A** and **B** in the micromolar range is significant because **A** and **B** exhibit cytostatic synergism against ELA carcinoma cells *in vitro* in the same concentration range (3). This suggests that PKC may be an ultimate target in the carcinoma cells for combinations of **A** and **B** and that **A** and **B** combine inside the carcinoma cells to form the potent PKC inhibitor **C**. It may be possible to enhance the potency of PKC inhibition for hydrazones related to **C** and the degree of synergism for combinations related to **A** + **B** through rational modifications in their chemical structures. We should stress, however, that the present studies do not exclude the possibility that **C** also acts on intracellular targets other than PKC to achieve its cytotoxic effects.

Under PKC assay conditions, when 200 μM **A** and 200 μM **B** are combined, the concentrations of **A** and **B** decrease by 33.8 ± 8.5 μM and 31.6 ± 9.2 μM, respectively, and the concentration of **C** increases by 38.1 ± 4.4 μM. These concentrations correlate with one another in a way that is consistent with a bimolecular reaction of **A** and **B** to form **C**. Hydrazone formation can follow pseudo-first-order or second-order kinetics, or a more complex intermediate kinetic order. The kinetic order depends on the relative rates of hydrazinocarbinolamine formation and dehydration (25). At low reactant concentrations, the reaction generally follows second-order kinetics. The second-order rate equation provided a somewhat better fit to the HPLC data depicted in Fig. 3 (r = 0.934) than did the first-order equation (r = 0.889). Although this suggests second-order kinetic behavior for reactions between 200 μM **A** and 200 μM **B**, the variability inherent in quantitative HPLC data makes it difficult to analyze the kinetic order with certainty.

The directly observed time-dependent decrease in PKC activity during exposure to 200 μM **A** + 200 μM **B** and the calculated curve (determined from HPLC kinetics of product formation and the dose–response relationship for inhibition of α-PKC by **C**) are consistent with one another, differing by no more than 10%. This consistency supports the hypothesis that the time-dependent drop in PKC activity is caused by an *in situ* reaction of **A** and **B** to form the more potent inhibitor **C** during the 1-hr incubation period.

The antiproliferative activity of phosphonium salt **C** (3) can be explained in terms of inhibition of PKC activity in the cytoplasm of ELA cells. Like tetraphenylphosphonium, **C** selectively inhibits ELA proliferation relative to untransformed cell proliferation, and it probably becomes concentrated selectively in ELA cytoplasm due to the abnormally high cytoplasmic membrane potential characteristic of these cells (3, 14, 22). Exposure to 7.9 μM **C** over a 48-hr period inhibits ELA cell growth by 50% (3). At this concentration, cytoplasmic **C** concentrations probably exceed 200 μM within 48 hr. This prediction is based on the observation that the related compound, tetraphenylphosphonium, achieves an intracellular-to-extracellular ratio of more than 80:1 within only 4 hr (3). Given the IC_{50} and m values for inhibition of ELA-derived α-PKC by **C** (20.4 μM and 1.2, respectively), the activity of α-PKC would be inhibited by 94% at 200 μM **C**. Although these data are consistent with the hypothesis that PKC is a significant target for **C** in ELA cells, further studies will be needed to ascertain the importance, if any, of PKC inhibition in the antiproliferative activity of **C** against mammalian cells.

In the absence of PtdSer, peptide substrate, and PKC, the rate of **C** formation from 200 μM **A** plus 200 μM **B** is relatively slow (Fig. 3). Concentrations of **C** reach 8.3 μM after 390 min in the absence of PtdSer, peptide substrate, and PKC as compared to 9.7 μM after only 15 min in the presence of these components. In other words, the PtdSer or peptides enhance the rate of formation of **C** by more than 20-fold. The rate observed in the absence of PtdSer and peptides is similar to the rate reported for the reaction between furfural and semicarbazide at 25°C, pH 7.0 (25). In contrast, the rate observed in the presence of PtdSer and peptides is unusually rapid. The higher rate in the presence of PtdSer and peptides is probably due to an increase in the effective concentrations of the cationic lipophilic molecules **A** and **B** due to their binding to PtdSer, an anionic surfactant: aggregates of anionic surfactants can enhance the rates of bimolecular reactions between pairs of cationic or lipophilic molecules by one to two orders of magnitude (26, 27). Like PtdSer *in vitro*, biomembranes in ELA carcinoma cells may enhance the rate of **C** formation from **A** and **B** inside the cells, contributing to the cytotoxic synergism in combinations of **A** and **B** (3).

We have developed hydrazone **C**, a PKC inhibitor active in the 10-μM range. This compound resembles existing inhibitors in being a dicationic lipophilic molecule with two delocalized charges, but it is otherwise structurally unique as a PKC inhibitor. Hydrazone **C** appears to exhibit greater cooperativity and slightly lower potency against the β_1 isoform as compared with the α isoform isolated from ELA carcinoma cells. Enzyme inhibition studies with single agents and combinations and HPLC studies of bimolecular reaction kinetics demonstrate that a combination of monocationic aldehyde **A** and monocationic acylhydrazide **B** can exhibit considerable synergism in their inhibition of PKC isolated from ELA cells through an unusual mechanism of interaction: direct covalent combination with one another to form a much more potent inhibitor. Inhibition of PKC may therefore be involved in the antiproliferative activity against ELA murine carcinoma cells of **C** and synergistic combinations of **A** and **B** (3). This work suggests the possibility that agents related to **C** could act as novel antineoplastic agents that exhibit preferential inhibition of particular PKC isoforms, selective accumulation in neoplastic cells, or both.

We thank Kevin O'Driscoll for performing the Western blot analysis and Jasmin Patel for reviewing this manuscript. This work was supported, in part, by National Cancer Institute Grants CA 44505 (T.C., J.S.J., and D.R.) and CA 02656 (S.A.R. and I.B.W.) and by PPG Industries (T.C., J.S.J., and D.R.). This is contribution no. 6461-MB from the Department of Molecular Biology in the Research Institute of Scripps Clinic.

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