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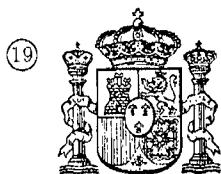
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NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

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R^4 , R^5 , R^6 y R^7 significan cada uno, independientemente, hidrógeno, halógeno, alquilo, hidroxilo, alcoilo, ariloxilo, haloalquilo, nitro, amino, acilamino, monoalquilamino, dialquilamino, alquiltio, alquilsulfonilo o alquilsulfonilo;

y uno de X e Y significa O y el otro significa O, S, (H, OH) o (H,H);

con la salvedad de que R^1 tiene un significado diferente de hidrógeno cuando R^2 significa hidrógeno, R^3 significa 3 - indolilo o 6 - hidroxil - 3 - indolilo, R^4 , R^5 y R^7 significan cada uno hidrógeno, R^6 significa hidrógeno o hidroxilo y X e Y significan ambos O y cuando R^2 significa hidrógeno, R^3 significa 3 - indolilo, R^4 , R^5 , R^6 y R^7 significan cada uno hidrógeno, X significa (H,H) e Y significa O; un grupo heterocíclico Het significa un grupo heterocíclico saturado, parcialmente insaturado o aromático, formado por 5 - 6 eslabones, puede tener en caso de desearse un anillo bencénico fusionado, pudiendo estar sin sustituir o sustituido por uno o varios sustituyentes del conjunto formado por halógeno, alquilo, hidroxilo, alcoilo, haloalquilo, nitro, amino, acilamino, mono - o dialquilamino, alquiltio, alquilsulfonilo y alquilsulfonilo; un grupo aromático heterocíclico R^3 es un grupo heterocíclico aromático Het tal como se definió antes; un grupo aromático carbocíclico R^3 significa fenilo o naftilo, que en caso de desearse puede estar sustituido igual que antes se indicó para el grupo Het; acilo solo o combinado es un grupo derivado de un ácido alcanoico con un máximo de 7 átomos de carbono de un ácido carboxílico aromático; alquilo solo o combinado significa un grupo alquilo lineal o ramificado con un máximo de 7 átomos de carbono; así como sales farmacéuticamente aceptables de compuestos ácidos de fórmula I con bases y de compuestos básicos de fórmula I con ácidos.

Objetos del presente invento son los compuestos antes definidos de por sí y como sustancias terapéuticamente activas; un procedimiento para su preparación; medicamentos que contienen dichos compuestos y la preparación de estos medicamentos; y el empleo de dichos compuestos en el control o prevención de enfermedades, especialmente de trastornos inflamatorios, inmunológicos, broncopulmonares y cardiovasculares, o para la preparación de un medicamento contra trastornos inflamatorios, inmunológicos, broncopulmonares y cardiovasculares.

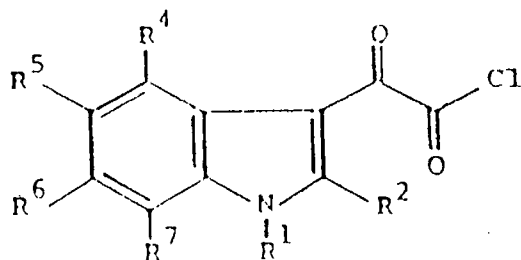
La invención se refiere además a un procedimiento de obtención de un medicamento cuyo principio activo, a saber uno de los compuestos definidos anteriormente, junto con un material soporte idóneo desde el punto de vista farmacéutico y demás auxiliares se convierte en una forma apta para fines terapéuticos, caracterizado por la obtención de un producto medicamentoso para el tratamiento de enfermedades inflamatorias, inmunológicas, broncopulmonares o cardiovasculares, producto que consta de la combinación de un preparado que contiene uno de los compuestos citados y de la información que guarda relación directa con el tratamiento de las enfermedades citadas.

En el marco de la invención el término "alquilo", solo o en combinación, significa un grupo de alquilo de cadena lineal o ramificada conteniendo un máximo de 7 átomos de carbono, de preferencia un máximo de 4, tal como metilo, etilo, propilo, isopropilo, butilo, butilo secundario, butilo terciario y pentilo. Ejemplos de grupos de alcoilo son metoxilo, etoxilo, propoxilo, isopropoxilo, butoxilo y t - butoxilo. Un grupo de haloalquilo puede comportar uno o más átomos de halógeno, siendo ejemplos de estos grupos clorometilo y trifluorometilo. La fracción de acilo de un grupo de acilamino, acilaminoalquilo o aciltioalquilo es la que deriva de un ácido alcanoico conteniendo un máximo de 7 átomos de carbono, de preferencia un máximo de 4 (por ejemplo acetilo, propionilo o butirilo) o de un ácido carboxílico aromático (por ejemplo benzóilo). El término "arilo", solo o en combinación tal como en arilsulfonilaminoalquilo, ariltioalquilo o aralquilo, significa un grupo de fenilo insustituido o un grupo de fenilo que comporta uno o más sustituyentes, de preferencia uno a tres, ejemplos de los cuales son halógeno, alquilo, hidroxilo, benciloxilo, alcoilo, haloalquilo, nitro, amino y ciano. El término "halógeno" significa flúor, cloro, bromo o yodo.

Ejemplos de grupos heterocíclicos Het son imidazolilo, imidazolinilo, tiazolinilo, piridilo y pirimidinilo.

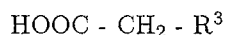
Un grupo aromático Het que contenga nitrógeno puede estar presente en forma de óxido de N. Ejemplos de grupos aromáticos carbocíclicos designados por R^3 son fenilo, 2 -, 3 - o 4 - clorofenilo, 3 - bromofenilo, 2 - o 3 - metilfenilo, 2,5 - dimetilfenilo, 4 - metoxifenilo, 2 - o 3 - trifluorometilfenilo, - 2, 3 - o 4 - nitrofenilo, 3 - o 4 - aminofenilo, 4 - metiltiofenilo, 4 - metilsulfonilfenilo, 4 - metilsulfonilfenilo, 4 - metilsulfonilfenilo y 1 - o 2 - naftilo.

El grupo aromático heterocíclico designado con R^3 puede ser un grupo 3 - indolilo, especialmente un grupo de la fórmula



VI

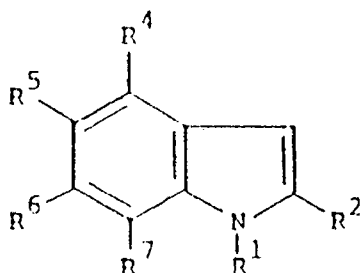
con un compuesto de la fórmula general



en donde R^1 a R^7 tienen el significado antes indicado, y, si se desea, modificando un sustituyente reactivo presente en un compuesto obtenido de fórmula II. Estas modificaciones funcionales pueden realizarse de igual modo que el descrito antes para la modificación funcional de un sustituyente reactivo presente en un compuesto de fórmula I.

La reacción de un compuesto de fórmula VI con un compuesto de fórmula VII se lleva a cabo, de preferencia en presencia de un agente aceptor de ácido, convenientemente una amina terciaria tal como trietilamina (por ejemplo trietilamina o diisopropiletilamina), y en un disolvente orgánico inerte tal como un hidrocarburo alifático halogenado.

Los compuestos de fórmula VI pueden prepararse haciendo reaccionar un compuesto de la fórmula general



VIII

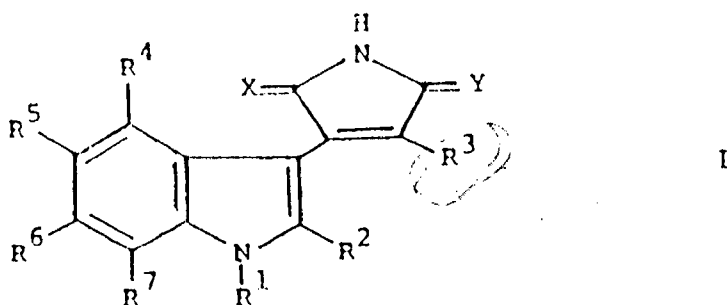
en donde R^1 a R^7 tienen el significado antes indicado, con un cloruro de oxalilo, convenientemente en un disolvente orgánico inerte tal como un hidrocarburo alifático halogenado, a una temperatura comprendida entre 0°C y la temperatura de reflujo del disolvente. El compuesto resultante de fórmula VI puede hacerse reaccionar in situ con el de fórmula VII o puede aislarse y purificarse antes de la reacción con el compuesto de fórmula VII.

Como se ha indicado antes, los compuestos de fórmula III se preparan, convenientemente, a partir de indol o un indol apropiadamente sustituido, o sea a partir de un compuesto de fórmula VIII en donde R^1 es hidrógeno, y un haluro de alquilmagnesio apropiado tal como bromuro o yoduro de metilmagnesio. Por ejemplo se puede tratar una solución del compuesto de fórmula VIII en un disolvente orgánico inerte tal como un hidrocarburo aromático con una solución etérea del haluro de alquilmagnesio o alrededor de la temperatura del ambiente.

Los compuestos de fórmula IV en donde R^8 tiene el mismo significado que R^3 antes indicado pueden prepararse bromando un compuesto de la fórmula general

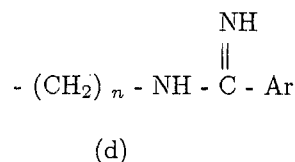
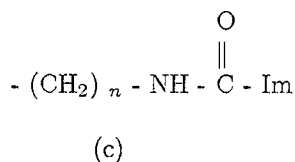
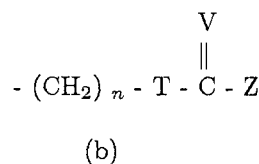
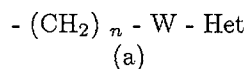
REIVINDICACIONES

1. Un procedimiento para la preparación de pirrol de la fórmula general



en donde

R^1 significa hidrógeno, alquilo, arilo, aralquilo, alcoxialquilo, hidroxialquilo, haloalquilo, aminoalquilo, monoalquilaminoalquilo, dialquilaminoalquilo, trialquilaminoalquilo, aminoalquilaminoalquilo, azidoalquilo, acilaminoalquilo, aciltioalquilo, alquilsulfonilaminoalquilo, arilsulfonilaminoalquilo, mercaptoalquilo, alquiltioalquilo, alquilsulfinilalquilo, alquilsulfonilalquilo, alquilsulfoniloxialquilo, alquilcarboniloxialquilo, cianoalquilo, amidinoalquilo, isotiocianatoalquilo, glucopiranosilo, carboxialquilo, alcoxicarbonilalquilo, aminocarbonilalquilo, hidroxialquiltioalquilo, mercaptoalquiltioalquilo, ariltioalquilo o carboxialquiltioalquilo o un grupo de la fórmula



en donde

Het significa un grupo heterocíclico,

W significa NH, S o un enlace,

T significa NH o S,

V significa O, S, NH, NO_2 , NCN o $CHNO_2$,

Z significa alquiltio, amino, monoalquilamino o dialquilamino,

Im significa 1 - imidazolilo,

Ar significa arilo, y

n tiene un valor de 2 - 6;

R^2 significa hidrógeno, alquilo, aralquilo, alcoxialquilo, hidroxialquilo, haloalquilo, aminoalquilo, monoalquilaminoalquilo, dialquilaminoalquilo, acilaminoalquilo, alquilsulfonilaminoalquilo, arilsulfonilaminoalquilo, mercaptoalquilo, alquiltioalquilo, carboxialquilo, alcoxicarbonilalquilo, aminocarbonilalquilo, alquiltio o alquilsulfinilo;

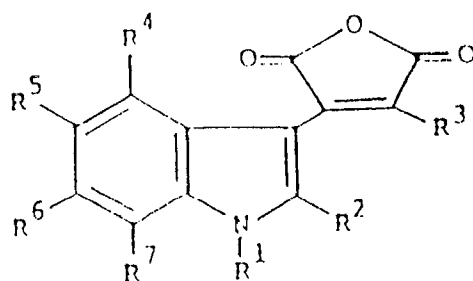
R^3 significa un grupo carbocíclico o heterocíclico aromático;

R^4 , R^5 , R^6 y R^7 significan cada uno, independientemente, hidrógeno, halógeno, alquilo, hidroxilo, alcoxilo, ariloxilo, haloalquilo, nitro, amino, acilamino, monoalquilamino, dialquilamino, alquiltio, alquilsulfinilo o alquilsulfonilo;

5 y uno de X e Y significa O y el otro significa O, S, (H, OH) o (H,H);

con la salvedad de que R^1 tiene el significado diferente de hidrógeno cuando R^2 significa hidrógeno, R^3 significa 3 - indolilo o 6 - hidroxil - 3 - indolilo, R^4 , R^5 y R^7 significan cada uno hidrógeno, R^6 significa hidrógeno o hidroxilo y X e Y significan ambos O y cuando R^2 significa hidrógeno, R^3 significa 3 - indolilo, R^4 , R^5 y R^7 significan cada uno hidrógeno, X significa (H,H) e Y significa O; un grupo heterocíclico Het significa un grupo heterocíclico saturado, parcialmente insaturado o aromático, formado por 5 - 6 eslabones, puede tener en caso de desearse un anillo bencénico fusionado, pudiendo estar sin sustituir o sustituido por uno o varios sustituyentes del conjunto formado por halógeno, alquilo, hidroxilo, alcoxilo, haloalquilo, nitro, amino, acilamino, mono - o dialquilamino, alquiltio, alquilsulfinilo y alquilsulfonilo; un grupo aromático heterocíclico R^3 es un grupo heterocíclico aromático Het tal como se definió antes; un grupo aromático carbocíclico R^3 significa fenilo o naftilo, que en caso de desearse puede estar sustituido igual que antes se indicó para el grupo Het; acilo solo o combinado es un grupo derivado de un ácido alcanoico con un máximo de 7 átomos de carbono de un ácido carboxílico aromático; alquilo solo o combinado significa un grupo alquilo lineal o ramificado con un máximo de 7 átomos de carbono; así como sales farmacéuticamente aceptables de compuestos ácidos de fórmula I con ácidos, **caracterizado** por

(a) para la preparación de un compuesto de fórmula I en donde X e Y significan ambos O, haciendo reaccionar un compuesto de la fórmula general



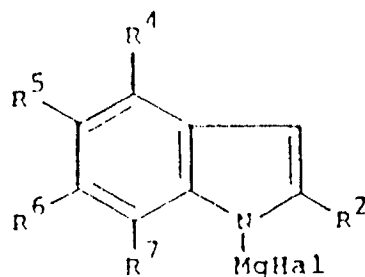
I I

en donde R^1 , R^2 , R^3 , R^4 , R^5 , R^6 y R^7 tienen el significado dado en la reivindicación 1,

40 como amoníaco bajo presión o con hexametildisilazano y metanol,

o bien

(b) para la preparación de un compuesto de fórmula I en donde R^1 significa hidrógeno y X e Y significan ambos O, haciendo reaccionar un compuesto de la fórmula general



I I I

en donde R^2 , R^4 , R^5 , R^6 y R^7 tienen el significado dado anteriormente y Hal significa halógeno, con un compuesto de la fórmula general



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United States Patent [19]**Schwartz et al.**[11] **Patent Number:** **5,821,072**[45] **Date of Patent:** **Oct. 13, 1998**[54] **COMBINATIONS OF PKC INHIBITORS AND THERAPEUTIC AGENTS FOR TREATING CANCERS**[75] Inventors: **Gary K. Schwartz**, Briarcliff Manor; **Anthony P. Albino**, New York, both of N.Y.[73] Assignee: **Sloan-Kettering Institute for Cancer Research**, New York, N.Y.[21] Appl. No.: **603,814**[22] Filed: **Feb. 20, 1996**[51] Int. Cl.⁶ **C12Q 1/48; C12N 9/10**[52] U.S. Cl. **435/15; 435/193; 435/194; 435/7.1; 435/7.23; 424/9.2**[58] Field of Search **435/194, 15, 7.23, 435/32, 193; 514/1; 424/9.2**[56] **References Cited****PUBLICATIONS**

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Attorney, Agent, or Firm—John P. White; Cooper & Dunham LLP

[57]

ABSTRACT

This invention provides methods for screening protein kinase C inhibitors capable of potentiating apoptosis in tumor cells. This invention also provides methods for screening antitumor therapeutic agents suitable for combination therapy with a protein kinase C inhibitors capable of potentiating apoptosis in tumor cells. This invention further provides different combination therapies comprising the specific protein kinase C inhibitors and the antitumor therapeutic agents.

3 Claims, 10 Drawing Sheets

potentiating apoptosis in tumor cells as determined by the above-described methods and a pharmaceutically acceptable carrier.

This invention provides a method for screening antitumor therapeutic agents suitable for combination therapy with a protein kinase C inhibitors capable of potentiating apoptosis in tumor cells comprising steps of: (a) contacting an amount of a protein kinase C inhibitor with tumor cells effective to potentiate apoptosis of tumor cells; (b) contacting the potentiated tumor cells of step (a) with an antitumor therapeutic agent; (c) determining the apoptosis of tumor cells; and (d) comparing the apoptosis determined in step (c) with apoptosis of same tumor cells which are only treated with the protein kinase C inhibitor, an increase in apoptosis indicating that the antitumor therapeutic agent is suitable for combination therapy with a protein kinase C inhibitor capable of potentiating apoptosis in tumor cells. In an embodiment, step (a) is carried out in the presence of the protein kinase C inhibitor. In another embodiment, the antitumor therapeutic agent is not previously known.

This invention also provides an antitumor therapeutic agent suitable for combination therapy with a protein kinase C inhibitors capable of potentiating apoptosis in tumor cells determined by the above-described methods.

This invention also provides a pharmaceutical composition comprising an effective amount of the antitumor therapeutic agent determined by the above-described methods, a protein kinase C inhibitor capable of potentiating apoptosis in tumor cells and a pharmaceutically acceptable carrier.

This invention provides a method for enhancing therapy in a tumor bearing subject comprising administering to the subject an effective amount of a specific protein kinase C inhibitor capable of potentiating apoptosis in tumor cells during or prior to the treatment of an antitumor therapeutic agent. This invention provides the above method, wherein the specific protein kinase C inhibitor is selected from a group consisting of Saffingol (L-threo-dihydrosphingosine), RO32-0432 (Bisindolylmaleimide tertiary amine), UCN-01 (7-OH-staurosporine), Flavopiridol (L86-8275), Bryostatins 1 (Macrocyclic lactone) and antisense nucleotides capable of inhibiting the expression of the protein kinase C. The above specific protein kinase C inhibitors are simply described for illustrative purposes of this invention. As it will be appreciated by a person of ordinary skill in the art that other specific protein kinase inhibitors may be used in this invention. Applicants have provided as methodology to determine whether a protein kinase C inhibitor may be useful for the claimed combination therapy.

In an embodiment, the antitumor therapeutic agent is a chemotherapeutic agent. In a further embodiment, the chemotherapeutic agent is Mitomycin C, Carboplatin, Taxol or Doxorubicin. In a still further embodiment, the antitumor therapeutic agent is a radiotherapeutic agent.

In an embodiment of the above-described methods, the tumor is a gastrointestinal cancer. In a further embodiment, the gastrointestinal cancer is gastric cancer, small bowel cancer, colon cancer or rectal cancer, leukemia or lymphoma.

In a separate embodiment of the above-described methods, the tumor is a breast cancer. In another embodiment, the tumor is an ovarian cancer. In a still another embodiment, the tumor is selected from a group consisting of prostate cancer, lung cancer, melanoma, cervical carcinoma, pancreatic cancer, sarcoma, hepatoma and gallbladder cancer.

Finally, this invention provides a method for potentiating apoptosis in tumor cells comprising contacting the cancer-

ous cells with an effective amount of a specific protein kinase C inhibitor capable of potentiating apoptosis during or prior to the treatment of an antitumor therapeutic agent.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of the Experiments

Materials and Methods

Cell culture

Early passage human gastric cancer cell lines SK-GT-5 and MKN-74 were established and characterized as described previously (13,14). All cultures were maintained at 37° C. in Eagles minimum essential media (MEM) supplemented with 20% fetal calf serum in an humidified 5% CO₂ atmosphere; the cultures tested negative for Mycoplasma species.

Determination of Apoptosis

Apoptosis was measured by two different methods: (i) Quantitative fluorescent microscopy (QFM) of nuclear changes induced by apoptosis, as determined by bisbenzimidide trihydrochloride (Hoescht-33258) staining of nuclear chromatin (15), and (ii) a terminal deoxynucleotidyl transferase (TdT) assay that labels the 3'-OH ends of DNA fragments produced in apoptotic cells (16). Cells were treated according to one of several protocols: (i) no drug (control) for 24 hours; (ii) safinol alone for 24 hours at 50 μ M, a concentration representing the highest non-toxic dose for SK-GT cells (as determined by cell proliferation studies with [³H]-thymidine (4)) and slightly exceeding the concentration (i.e., 30 μ M) which inhibits PKC enzyme activity by 50% in vitro; (iii) Mitomycin-C alone at 5 μ g/ml for 24 hours; (iv) a combination of safinol (50 μ M) and Mitomycin-C (5 μ g/ml) for 24 hours; (v) safinol (50 μ M) alone for 1 hour, followed by an immediate wash out of the drug with fresh MEM, and then an additional 24 hours of MMC (5 μ g/ml) exposure; (vi) safinol (50 μ M) with 1 ng/ml 3-phorbol 12-myristate 13-acetate (PMA) for 1 hour, followed by an immediate wash out of the drug with MEM and 24 hours of MMC (5 μ g/ml) exposure; and (vii) either safinol (50 μ M) alone or PMA (1 ng/ml) alone for 1 hour, followed by drug washout with MEM and 24 hours of exposure to media without drug. The dose of MMC used was based on proliferating studies with [³H]-thymidine (14, and unpublished data) indicating 20% inhibition of cell proliferation. MMC was diluted in water. A stock safinol solution was constituted in DMSO and was subsequently diluted at least 1:100 in media for all experiments. This dilute concentration of DMSO does not induce apoptosis or inhibit cell proliferation in these cell lines (unpublished data). For QFM determinations, the cells were fixed in 3% paraformaldehyde and incubated at room temperature for 10 minutes. The fixative was removed and the cells were washed with 1 $\times$ PBS, resuspended in 20 μ l 1 $\times$ PBS containing only 8 μ g/ml of bisbenzimidide trihydrochloride (Hoechst #33258), and incubated at room temperature for 15 minutes. Aliquots of the cells (10 μ l) were placed on glass slides coated with 3-amino-propyl-triethoxysilane, and duplicate samples of 500 cells each were counted and scored for the incidence of apoptotic chromatin condensation using an Olympus BH-2 fluorescence microscope equipped with a BH2-DM2U2UV Dich. Mirror Cube filter (Olympus, Lake Success, N.Y.). For the TdT assays, the ApopTag Kit (Oncor, Gaithersburg, Md.) was used. This method employs a fluoresceinated anti-

activity to determine the absolute radioactive incorporation attributable to cdk2. The samples were transferred to a 10% SDS PAGE gel for analysis. The autoradiographs were analyzed on a phosphorimager for determinations of relative cdk2 activity. The results are shown in FIG. 8

Each lane represents the following: lane A, the IgG control; lane B, no drug therapy for 24 hours; lane C, MMC alone (5.0 $\mu\text{g/ml}$) for 24 hours; lane D, safingol alone (50 μM) for 24 hours; lane E, the combination of safingol (50 μM) and MMC (5.0 $\mu\text{g/ml}$) for 24 hours. The results indicate that untreated gastric cancer cells (lane B) have increased cdk2 activity and that both MMC (lane C) and safingol (lane D) have decreased cdk2. However, the combination of MMC and safingol increased cdk2 activity back to basal levels.

These results may explain why safingol, as a single agent, does not induce apoptosis, but for apoptosis to proceed in the presence of safingol there is an obligate requirement for concomitant exposure of the tumor cells to a chemotherapeutic agent (e.g. MMC). Applicants propose that apoptosis takes place when at least three conditions are met: DNA damage by chemotherapy, an increase in cdk2 activity, and inhibition of PKC activity. Thus, in control cells there is an increase in cdk2 activity but no DNA damage or PKC inhibition; in MMC treated cells there is DNA damage, a decrease in cdk2 activity, and no PKC inhibition (data not shown); and in safingol treated cells there is PKC inhibition but a decrease in cdk2 activity and no known DNA damage. However, with the combination therapy of safingol and MMC, all three conditions are met: increased cdk2 activity, DNA damage, and inhibition of PKC. Thus, apoptosis proceeds. The basis for the decrease in cdk2 activity by safingol (as well as by MMC) remains unknown and stands in contrast to the effects of UCN-01 and bryostatin as single agents. However, flavoperidol alone has been reported to inhibit the cdk2s (39), including cdk2 (Edward Sausville, NCI, Bethesda, Md.), at concentrations in which applicants have observed flavopiridol to inhibit PKC activity. All these results strongly suggest that cdk2 activity may be a major marker for the apoptosis associated with PKC inhibition. The increase in cdk2 activity with safingol and MMC, indicates a potential biomarker of apoptosis that will be tested in human tissues on applicants' clinical trials (see METHODS and PLANS below).

Safingol enhances the anti-tumor effect of cisplatin and doxorubicin in tumor-bearing animals

Although safingol showed no direct anti-tumor activity in vivo, when tested as a single agent in a variety of murine tumor models and human tumor xenografts, its synergism with cisplatin and doxorubicin in vitro suggested it might potentiate the antitumor efficacy of these chemotherapeutic agents in vivo. For these experiments safingol was administered alone or in combination with cisplatin or DOX to female C3H/HeJ mice bearing 16C mammary carcinoma implanted intramuscularly. Antitumor response to the agents alone or in combination was assessed by tumor growth delay. Safingol by itself at doses of 5 to 20 mg/kg had no effect on inhibiting tumor cell growth. However, when administered intraperitoneally (i.p.) at a dose of 20 mg/kg and combined with intravenous (i.v.) cisplatin (10 mg/kg), safingol produced a modest enhancement in growth delay when compared to cisplatin alone. The administration of cisplatin under these conditions resulted in the development of a 16C mammary carcinoma that took 10 days to reach 5 times its starting size, whereas the combination of the same dose of cisplatin and a single dose resulted in a tumor mass that took 26 days to reach 5 times its starting

size. Comparable effects against 16C tumors were observed with safingol and DOX (10 mg/kg, i.v.) (74).

Safingol does not induce increased toxicity of chemotherapy in animals

From the preclinical data it appears that doses of safingol ranging from 10 to 20 mg/kg are necessary in order to achieve any level of chemopotentialization. Because safingol is being developed as a chemopotentialization agent, studies were conducted in rats and dogs to evaluate the ability of safingol to potentiate the toxicity of established cancer chemotherapeutic agents (40). Single-dose studies with rats in which safingol was administered prior to cisplatin, cyclophosphamide, or doxorubicin showed that safingol did not potentiate the myelotoxicity of these agents or affect reversal of the toxicity. No overt potentiation of cisplatin-mediated nephrotoxicity was observed. Cisplatin had no apparent effect on the pharmacokinetic profile of safingol. A possible effect of safingol on cisplatin pharmacokinetics was demonstrated by a statistically significant higher cisplatin Cmax value following safingol treatment, compared with the cisplatin Cmax value following vehicle administration. Other than the Cmax time point, the concentration-time profile of cisplatin was unaffected by safingol. A single-dose combination study in dogs indicated that safingol did not potentiate doxorubicin toxicity and vice versa, and that plasma concentrations of safingol or doxorubicin were also unaffected by administration of the other (Investigators brochure).

Intravenous administration of safingol in emulsion caused clinical pathology and histopathology findings consistent with venous irritation and transient intravascular hemolysis in mice, rats, and dogs (40). It seemed to occur as a function of safingol concentration and depended on the caliber of the vein used for delivery. Although venous irritation and hemolysis were significant and, in some instances, dose limiting toxicities in the animal studies, it was demonstrated that these effects may be mitigated by the use of low safingol concentrations and large-sized, high flow veins.

Results from the phase I clinical trial combining safingol with doxorubicin:

(i) Study design: This Phase I trial was designed as an open-label, non-randomized, dose escalation study, in which groups of three to six patients received on day 1 of each cycle received sequentially increased dosages of intravenous safingol emulsion and then (provided there was no acute toxicity) 14 days later received the same dose of safingol one hour prior to a fixed intravenous dose of doxorubicin. Individual patients could receive up to six cycles of treatment with the same dose of safingol and doxorubicin (DOX) every 21 days, until signs of tumor progression or unacceptable toxicity occurs, or until the patient's cumulative life-time dose of doxorubicin exceeds 400 mg/m² with an additional cycle of treatment. Prior DOX therapy was allowed up to a dose of 280 mg/m² as long as the left ventricular ejection fraction was >50%. The starting dose of safingol was 15 mg/m². This represented 1/10 the LD₅₀ in mice, 4% the highest non-toxic dose (HNTD) in dogs, and 12% the HNTD in rats.

(ii) Patient characteristics: To date applicants have entered 17 patients onto the study, all of whom are evaluable. The patient characteristics are as follows: 1) median age (range): 59 (29-77), 2) median KPS (range): 80 (70-90) 3) Male:Female : 10:7, 4) Primary sites of disease: pancreas:6, gastric:2, colon:3, unknown primary:2, sarcoma:3, nasopharyngeal:1;

(iii) No dose limiting hematologic Toxicity: As shown in the table below with a fixed doxorubicin dose of 45

induction of apoptosis. Selective PKC inhibitors other than safinol and UCN-01 may induce even greater degrees of apoptosis when combined with chemotherapy against breast (MDA-MB-468), gastric (SK-GT and MKN-74) and ovarian (OVCAR) cancer cells. The other PKC inhibitors to be tested for the induction of apoptosis against the cell lines including a series of Roche inhibitors (to be supplied by Roche Pharmaceuticals, Nutley, N.J.), and bryostatin 1 (36), an agent that activates PKC with short exposure but inhibits PKC following prolonged drug exposure by inducing PKC degradation. Apoptosis will be quantitated by two methods: (1) QFM staining: This method involves staining with bisbenzimid trihydrochloride (Hoescht-33258) of condensed chromatin which characterizes the cells undergoing apoptosis (3,5) and (ii) terminal deoxynucleotidyl transferase (TdT) assay which labels the 3'-OH ends of DNA fragments in apoptotic cells (5,43).

For QFM determinations, the cells are fixed in 3% paraformaldehyde and incubated at room temperature for 10 minutes. The fixative is removed and the cells are washed with 1x PBS, resuspended in 20 μ l 1x PBS containing only 8 μ g/ml of bisbenzimid trihydrochloride (Hoechst #33258), and incubated at room temperature for 15 minutes. Aliquots of the cells (10 μ l) are placed on glass slides coated with 3-amino-propyl-triethoxysilane, and duplicate samples of 500 cells each were counted and scored for the incidence of apoptotic chromatin condensation using an Olympus BH-2 fluorescence microscope equipped with a BH2-DM2U2UV Dich. Mirror Cube filter (Olympus, Lake Success, N.Y.).

For the TdT assays, the ApopTag Kit (Oncor, Gaithersburg, Md.) will be used. This method will essentially be identical to the one described above for tissue and lymphocyte samples from patients except that after washing with 0.1% Triton X-100 the cells will be counterstained with propidium iodide (PI) solution. Green (d-dUTP labeled DNA strand breaks) and red (PI staining for total DNA content) fluorescence of individual cells are measured on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). The resulting bivariate plots enables the detection of apoptotic events within the cell cycle. The R_1 cursor is set using the control specimen to define normal levels of green fluorescence (i.e., basal levels of apoptosis). Cells with fluorescence above the R_1 cursor are considered apoptotic. The data from 10,000 cells are collected and analyzed using CellFit and LYSYS software (Becton Dickinson).

(ii) To define the optimal conditions in which to combine safinol, UCN-01, flavoperidol, and bryostatin with chemotherapy for future clinical development: Safinol, UCN-01, and flavoperidol are now in phase I clinical trials. Combining these agents with chemotherapeutic agents appears to be the best way to maximize the beneficial effects of these drugs as anti-cancer agents against breast cancer cells. However, the optimal way in which to achieve this end remains to be defined. Applicants plan to pursue this issue with a series of studies:

The optimal chemotherapeutic agent(s): The plan is to perform experiments with safinol and UCN-01 in combination with other conventional chemotherapeutic agents such as cis-platin, Taxol, 5-fluorouracil and doxorubicin in order to determine the extent to which these agents induce apoptosis with these other agents in the breast (MDA-MB-468), gastric (SK-GT and MKN-74) and ovarian (OVCAR) cells. The methods to quantitate apoptosis will be by QFM and TdT assays, as described above.

Combinations of PKC inhibitors: Since many of these inhibitors act at different sites of the enzyme (regulatory vs.

catalytic), it may be possible to combine multiple PKC inhibitors together with chemotherapy in order to further enhance the induction of apoptosis. For example, as applicants have shown, the combination of UCN-01 and safinol with MMC at the conditions described above induce a greater degree of apoptosis than treatment either PKC inhibitor alone with MMC. Further studies combining flavoperidol with safinol or with UCN-01 are planned and apoptosis will be quantitated by the QFM and the TdT technique.

The optimal sequence and timing of the PKC inhibitors with the chemotherapeutic agent: The exact sequence and timing of the PKC inhibitor relative to chemotherapy exposure in breast cancer cell remains unknown. For these experiments applicants propose exposing the breast and gastric cancer cells to safinol (50 μ M) for different time intervals (30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, and 24 hours), washing the cells free of drug, and then exposing to MMC (5 μ g/ml) for 24 hours. The relative induction of apoptosis will then be quantitated as above. Conversely, cells will first be exposed to safinol for 24 hours, but the MMC exposure will now be limited to different time intervals before being assayed for apoptosis.

(iii) To examine in vitro cdk2 and to identify other related cell cycle dependent proteins associated with the induction of apoptosis and the inhibition of PKC: The basis for the increase in cdk2 activity by safinol and MMC in combination remains unknown. It may be related to a decrease in the expression of a cdk2 inhibitor (i.e. p21) or it may be related to a modification of a phosphorylated site on cdk2 which results in its activation. A further understanding of this process may lead to new surrogate markers of activity. In order to examine this further applicants plan to examine: 1) protein expression of p21 and p27 with immunoblotting using an enhanced chemiluminescence system using specific antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, Calif.); 2) differences in protein phosphorylation of cdk2 with [^{32} P]orthophosphate cell labelling, as previously described, using a cdk2 specific antibody suitable for immunoprecipitating (36); and 3) correlative studies of cdk2 activity as measured by the histone H1 kinase assays (38). Control cells will be treated in the same way as described above except that standard media without drug will be used for all incubations.

To determine whether a specific PKC isoform may be a critical target for drug development in induction of apoptosis with chemotherapy by: (i) testing whether antisense for PKC α in combination with MMC has superior anti-tumor activity against human gastric cancer cells when compared to either agent alone or to a PKC missense with MMC; (ii) testing other PKC antisense against other PKC specific isoforms in combination with chemotherapy; (iii) studying in gastric cancer cells whether any of the PKC isoforms are being preferentially inhibited during the induction of apoptosis.

(i) Testing whether antisense for PKC α in combination with MMC has superior anti-tumor activity against human gastric cancer cells when compared to either agent alone or to a PKC missense with MMC: applicants' results indicate that PKC represents a novel target for enhancing the induction of apoptosis by chemotherapy. However, PKC is ubiquitous in both normal and malignant tissue. Even though safinol's specificity represents a considerable advance in the development of PKC inhibitors especially as they pertain to eventual clinical development, even further specificity would still provide theoretical advantages in drug development.