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13. ABSTRACT
Sigma-2 Receptors are highly expressed in many tumor cell lines. Treatment with sigma-2 selective (CB184, CB64D) and non-selective agents (haloperidol, reduced haloperidol) produces cell death by a mode consistent with apoptosis. Apoptosis was confirmed by the TUNEL assay and Annexin V binding. Cell death was quantified by measuring lactate dehydrogenase (LDH) into culture media. Sigma 2 selective agents CB64D and CB184 possess similar potency in MCF-7 breast tumor cells and breast tumors with mutations in the p53 tumor suppressor gene that are phenotypically resistant to certain anti-neoplastic agents (MCF-7/Adr-, T47D, SKBr3). Doxorubicin and Actinomycin D cytotoxicty are abrogated by inhibitors of caspases (Z-VAD-FMK, Y-VAD-CHO, DEVD-CHO), whereas sigma-2 agonist cytotoxicity is unaffected by caspase inhibitors (Annexin V binding, LDH release assays). The Sigma-2 agonist CB184 can potentiate cytotoxicity of doxorubicin and actinomycin D in MCF-7 cells and MCF-7/Adr- cells. Haloperidol and pentazocine (racemic) potentiate doxorubicin cell killing in MCF-7/Adr- cells. These data suggest different pathways are involved in sigma-2 mediated cytotoxicity as compared with DNA-damaging anti-neoplastics. Further, sigma agonists may have a role in the clinical management of breast cancer, particularly in drug-resistant tumors.

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FOREWORD

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Keith W. Crandell 6-3-99

PI - Signature Date

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Introduction

Work for the revised grant entitled *Sigma Receptors and Apoptosis in Breast Tumor Cells* began in August of 1998 in the laboratory of Dr. Wayne Bowen, Chief of the Section of Receptor Biochemistry and Pharmacology, Laboratory of Medicinal Chemistry , NIDDK. Our lab, as well as others, had observed the sigma receptors are expressed in high densities in a variety of tumor cells, including breast tumors. Chronic exposure to Sigma agonists diminishes cell proliferation, induces certain morphological changes and ultimately results in the death of the cell. The purpose of my work has been to characterize sigma-2 mediated effects in breast tumors and elucidate biochemical pathways involved in the effects. The scope of work will determine the ability of sigma receptors to modulate the action of pro-apoptotic (Bax, BAD) or anti-apoptotic proteins (bcl, bcl_x). Various genes that are activated or suppressed during sigma receptor-signalling will also be explored. Here, we report our initial findings characterizing the effect of different sigma receptor agonists in a variety of breast tumors, and contrast their actions with those of clinically available anti-neoplastic agents.

Training Accomplishments

The training experiences have included a diverse array of formats from didactic courses to research seminars to methodology workshops. Some of these sessions expand my knowledge about basic processes involved in carcinogenesis, while others instruct state-of-the-art techniques designed to elucidate mechanisms leading to carcinogenesis. I completed a course on clinical research design offered through the National Institutes of Health Clinical center. This totally comprehensive course presented all aspects of research design including statistical and methodological issues, legal issues, preparing a budget and resource management. Many of the lectures were directly relevant to cancer clinical research and the information disseminated was very practical.

At the annual meetings of the Society of Toxicology (SOT) and the American Association of Cancer Research (AACR), I attended methods workshops particular useful in assessing the involvement of target genes and proteins in pharmacologic and toxicologic responses. The sessions on gene microarray methodology will be utilized in the upcoming year as I continue with my research objectives of elucidating the mechanism of sigma 2-receptor mediated cytotoxicity in tumors. Symposia and poster sessions attended at both these conferences, as well as numerous lectures attended at NIH and Howard University have tremendously expanded my knowledge base in areas including apoptosis, cell-cycle control in carcinogenesis, the role of p53 tumor suppressor in cancer, and mechanisms of anti-neoplastic drug resistance. These presentations are ideal settings for interfacing with researchers addressing similar research questions as my own.

Research Accomplishments

As of this date, the research objectives in my revised proposal (Sigma 2 Receptors and Apoptosis in Breast Cancer Cells) is progressing remarkably well and as projected in the

proposal. My work up to this point was presented at the AACR meeting in Philadelphia, Pa., April 10-14 (see abstract in appendix). As a result of my work, I received an award for excellence as a faculty researcher by the AACR (see award letter in appendix). We have prepared a manuscript of this work that is being submitted for publication. A copy of the manuscript is included in the appendix. The following is a summary of the data presented in the manuscript.

Sigma 2 Receptor Agonists Produce Death in Breast Tumors by a Mechanism Consistent with Apoptosis

I have demonstrated the ability of different Sigma 2 selective and non-selective agonists on cell killing in a variety of Breast Tumors. Tumor Cell lines used include MCF-7 cells, and 3 cell lines possessing p53 mutations (MCF-7/Adr-, T47D, SkBr3), some of which are resistant to anti-neoplastic agents. The MCF-7/Adr- line is resistant to doxorubicin..

The nuclear alterations and DNA cleavage that a characteristic of apoptosis are induced by sigma-ligands and DNA-damaging agents such as doxorubicin, as evidenced by the TUNEL assay. Yet these changes occur on exposure to sigma-ligands in cell lines that are resistant to doxorubicin, suggesting that different pathways are involved in cell-killing by these different agents Morphological changes of apoptosis are mediated by caspases. We compared the effect of caspase inhibition in sigma-mediated cytotoxicity with that induced by doxorubicin and actinomycin D. Co-exposure to caspase inhibitors and doxorubicin, assessed by annexin staining and LDH release, suggest a mechanism of cell death with some changes consistent with apoptosis and some consistent with necrosis. Actinomycin D cytotoxicity measured by LDH release, at higher doses, is totally abolished by inhibition of caspase 3., a central activation point of multiple caspase pathways. Sigma-2 cytotoxicity is predominantly unresponsive to inhibition by caspases as assessed by Annexin V binding and LDH release.

We have observed the ability of sigma-2 selective ligands to potentiate the toxicity of doxorubicin and actinomycin D in MCF-7 cells and MCF-7/Adr- cells. At concentrations of CB184 that are non-toxic, combining the agents profoundly increases the extent of anti-neoplastic cell-killing, and reduces the time to observe maximal effects.

In MCF-7/Adr- cells, but not MCF-7 cells, the non-selective sigma agonists haloperidol and pentazocine (racemic) also display the potentiation phenomenon. The clinical availability of these drugs suggest a possible therapeutic application whereby drug resistant-tumors can be treated and toxicity minimized.

Key Research Accomplishments

1) Sigma-2 selective and non-selective receptor agonists are toxic to a variety of breast tumors irrespective of p53 genetics and/or drug-resistance phenotypes
2) Based on TUNEL assay and Annexin V binding assay, the mechanism of sigma-2 mediated cytotoxicity is consistent with apoptosis
3) In studies involving the use of caspase inhibitors, the mechanism of cell death appears to be independent of caspase-pathways

4) Sigma agonists can potentiate cytotoxicity of anti-neoplastic drugs, particularly in drug-resistant cell lines.

Reportable Outcomes

- an abstract is published in the AACR 1999 conference booklet. This data was presented at the meeting in Philadelphia, March 10-14. A manuscript has been prepared and submitted (abstract and manuscript draft are included in the appendix)
- I have received an AACR Faculty award for excellence in research based on my work presented at the meeting
- An employee invention report (EIR) has been submitted to the NIH based on our finding the sigma agonists potentiate anti-neoplastic agents (#E-165-9910, *Sigma-2 Receptor Ligands Potentiate the Activity of Anti-neoplastic Drugs in Tumors with Wild-Type and Mutant p53 Tumor Suppressor Genes*). The EIR is a preliminary step before a patent can be submitted. The technology or invention must be researched for the existence of any prior patent filings.
- A book chapter entitled *Genetics and Cancer* was co-authored with Dr. Peter Shields and has recently been published (Mark Miller, editor).

Sigma-2 receptor activation induces apoptosis in breast, prostate, and neuroblastoma cell lines. Crawford, K.W., Vilner, B.J., and Bowen, W.D. *Lab. Medicinal Chemistry, NIDDK, NIH, Bethesda, MD 20892.*

We have shown that sigma-1 and sigma-2 receptors are highly expressed in many tumor cell lines (*Can. Res.* 55: 408, 1995) and that treatment of cells with sigma receptor ligands causes dose- and time-dependent changes in cell morphology (which include loss of processes, rounding, detachment) and cell death (*J. Neurosci.* 15:117, 1995) . Here we show that the mode of cell death is apoptotic. Cells were treated for various time periods with various sigma receptor ligands (reduced haloperidol, BD737, CB-64D, (+)-pentazocine). Apoptosis was determined by TUNEL or by Annexin-V binding. Cell death was quantitated by release of LDH or nuclear matrix protein. Treatment with sigma-2 receptor ligands (0.1 - 100 μM) for up to 3 days, resulted in apoptosis in MCF-7 breast adenocarcinoma, T47D breast ductal carcinoma, SK-N-SH neuroblastoma, and DU-145 prostate carcinoma. Sigma-1 receptor-selective ligands had no effect. The sigma-2 selective agonist CB-64D produced nearly the same cytotoxicity in "wild-type" MCF-7 cells as in drug-resistant breast cell lines possessing p53 mutations; MCF-7-Adr^r (adriamycin-resistant) and SKBr3 (actinomycin-D-resistant). Furthermore, in preliminary studies, the caspase inhibitor Z-VAD-FMK abolished doxorubicin induced cytotoxicity and apoptosis in MCF-7 cells, but had no effect on the actions of CB-64D. These data suggest that sigma-2 receptor activation and DNA damaging agents induce apoptosis via different signalling mechanisms. Sigma-2 receptors appear to be novel components of the apoptotic pathway, and may represent useful targets for antineoplastic agents, particularly in treating chemotherapy-resistant tumors.

Proceedings of The Ammerican Association for Cancer Research; Abstract#1104

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MARGARET FOTI, Ph.D.,
EXECUTIVE DIRECTOR

February 5, 1999

Dr. Keith W. Crawford
Instructor, Department of Pharmacology
Howard University
College of Medicine, Room 3409
520 W St., N.W.
Washington, DC 20059

Dear Dr. Crawford:

It is with pleasure that I write to congratulate you on being selected as one of the recipients of the 1999 AACR-HBCU Faculty Award in Cancer Research. The American Association for Cancer Research is very pleased to administer this important program, which is supported by a generous grant from the Comprehensive Minority Biomedical Program of the National Cancer Institute (NCI) and provides funds for the participation of meritorious faculty of Historically Black Colleges and Universities in the 90th Annual Meeting of the Association, April 10-14, 1999, in Philadelphia, PA. I am pleased to inform you that your application was judged by the Award Selection Committee to be highly deserving of this meritorious Faculty Award.

The award will consist of financial support for your participation in the meeting. You will be reimbursed for travel, hotel expenses, and subsistence, up to $1,500, pending receipts of these expenses. Reimbursement is contingent upon receipt of a written report commenting on the scientific sessions you attended at the annual meeting and the AACR-HBCU Faculty Award Program. Faculty members who receive this award are expected to attend the 1999 Annual Meeting for the duration of the meeting, *i.e.*, four days, and are expected to participate in all planned events for awardees. Please complete the attached AACR Expense Report Form (receipts for these expenses are required), and return it with your report on the Conference to the attention of Ms. Robin E. Felder, Membership Development Coordinator, no later than **May 21, 1999.** Reimbursement will be issued promptly upon the receipt of your request.

In order to receive this award we must receive certification from you **no later than February 12, 1999,** verifying your eligibility for and acceptance of this award. Because of the size of this award and the limited funds provided for this purpose, the NCI mandates that no indirect costs may be deducted by your institution. **Please return the enclosed Certification of Status Form by Friday, February 12, 1999, in order to expedite the processing of this award.**

Enclosed you will find the *Second Announcement* for the Annual Meeting. This booklet contains a preliminary scientific program, information concerning travel, as well as forms for registration and housing. If you have not yet made your hotel or travel arrangements to Philadelphia, we recommend that you do so as soon as possible. If you need assistance with your travel arrangements you may contact our official travel agent, Association Travel Concepts at (1-800-278-1140). Association Travel Concepts will be happy to assist you with your arrangements.

The Association is committed to increasing participation in its activities by scientists from Historically Black Colleges and Universities, and to encouraging faculty and their students to pursue careers in cancer research. If you are not currently a member of the American Association for Cancer Research, we would be pleased to activate your Active Membership, effective immediately, and waive your dues payment for 1999. By joining the Association, you will have early access to the most up-to-date scientific findings, information on professional careers in the cancer field, and networking opportunities with senior scientists who have established themselves in positions within academia,

government, and industry. Enclosed you will find information relevant to AACR membership. To activate your membership immediately, you should send a brief letter indicating your desire to join the AACR, and complete the attached new member form.

Please be aware that several functions have been organized for Awardees during the annual meeting. As a recipient of this award, it is mandatory that you participate in all of the following activities:

! A Welcoming Dinner will take place on Saturday, April 10, 1999, from 6:30 p.m. to 8:30 p.m., in Room 201A of the Pennsylvania Convention Center. Members of the Award Selection Committee, NCI officials, and members of the AACR Board of Directors will participate in this event. You will also be provided with materials to facilitate your participation in the meeting, and will have the opportunity to obtain answers to any questions.

! The AACR Minority Issues Committee has organized an exciting symposium entitled, *"Reaching Back to Move Forward: Mentoring and the Budding Scientist,"* which will be held on Sunday, April 11, 1999, from 6:30 p.m. to 8:30 p.m. in Salons A-D of the Philadelphia Marriott Hotel. During this symposium, 1999 AACR Awardees will be asked to stand and be recognized. This event has been a regular feature of the AACR meeting since 1990 and has proven to be very valuable to those who attend.

! A Reception will take place on Tuesday, April 13, 1999, from 7:00 p.m. to 8:30 p.m., in Salon H of the Philadelphia Marriott Hotel. This will be an opportunity to discuss your observations of the scientific program with key AACR members and selected NCI staff members. Awardees from previous years as well as senior scientists who have made major presentations at the meeting have been invited to participate and share their extensive knowledge and experience with the Awardees.

Finally, regulations require that you provide a comprehensive report on your attendance at the meeting; these reports are essential to the ongoing evaluation of the success of this program by both the AACR and the NCI. Your report should include detailed comments on the scientific program of the annual meeting as well as on the AACR-HBCU Faculty Award Program. We ask that you address the following issues in your report: 1) how you learned about this program; 2) how the receipt of this Award contributed to your attendance at the meeting; 3) how you benefited by attending the organized functions for Awardees; 4) which scientific sessions you attended and found most helpful; and 5) what was gained through your interactions with junior and senior scientists during the meeting, *e.g.*, renewal of friendships, establishment of contacts for future scientific collaborations, etc. **This report should be received by the AACR no later than May 21, 1999.**

Thank you in advance for your prompt response to the deadline of February 12, 1999. We look forward to hearing from you and to seeing you at the meeting. Please contact Ms. Robin E. Felder, Membership Development Coordinator, or Ms. Ivelys Figueroa, Administrative Assistant, Membership, at (215) 440-9300 if you have any questions about this program.

Again, our best wishes on this honor. We are delighted about your interest in the AACR and its annual meeting.

Sincerely,

Margaret Foti, Ph.D.
Executive Director

MF:rf
Enclosures
- Certification Form
- Annual Meeting *Second Announcement*
- Active Membership Form
- Guidelines for Reimbursement and Reimbursement Form

cc: Dr. Bayard D. Clarkson, Treasurer, AACR
Dr. Sanya Springfield, Program Director, Comprehensive Minority Biomedical Program, NCI

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Keith W. Crawford, Ph.D.

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OCTOBER 13, 1998 TO MARCH 16, 1999

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JOHN I. GALLIN, M.D.
DIRECTOR, NIH CLINICAL CENTER
NIH ASSOCIATE DIRECTOR FOR CLINICAL RESEARCH

APRIL 19, 1999

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Sigma Receptor Agonists Induce Apoptosis in Chemo-resistant Breast Tumors by a Caspase-Independent Mechanism

Keith W. Crawford* and Wayne D. Bowen+

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+ Unit on Receptor Biochemistry and Pharmacology, Laboratory of Medicinal Chemistry, NIDDK

INTRODUCTION

Sigma receptors are unique drug binding proteins which are present in the central nervous system as well as in various peripheral tissues (1). They recognize a variety of psychoactive agents from various structural classes, including opioids, like pentazocine, and possess high affinity binding of neuroleptic drugs such as haloperidol. There is strong evidence for endogenous sigma receptor ligands, and although progesterone and other neurosteroids have been suggested as candidates (2), none has yet been conclusively identified. The functions of these receptors are not yet clearly defined. In the central nervous system, they have been shown to be involved in regulation of neurotransmitter release, modulation of neurotransmitter receptor function, learning and memory processes, and regulation of movement and posture. Though present in peripheral tissues such as liver, kidney, and endocrine organs, functions in these tissues is much less understood.

Two subtypes of sigma receptor have been identified, termed sigma-1 and sigma-2 (3-5). The subtypes are distinguishable pharmacologically, functionally, and by molecular size. Sigma-1 receptors have been cloned and shown to be distinct from any known receptor class (6). The sigma-1 receptor is a 25 kDa, single polypeptide with one putative transmembrane region. The sigma-2 receptor is an 18 - 21 kDa protein, but has not yet been cloned (3, 5).

We have shown that both sigma receptor subtypes are highly expressed in tumor cell lines from various tissues (7). Interestingly, sigma receptors are more highly expressed in rapidly proliferating cells and are down-regulated when cells become quiescent (8). In the human breast, sigma receptors were virtually absent in normal tissue, but were present in high density in breast tumor biopsy tissue (9). Their high density in various tumor cell types, and particularly in proliferating cells, makes sigma receptors potential targets for diagnostic imaging as well as therapeutic agents (8, 10).

We have demonstrated that treatment of cells with sigma receptor agonists causes dose- and time-dependent changes in cell morphology, which include loss of processes, rounding, and detachment from substratum (11). Continued exposure of cells to sigma ligand results in cell death. In addition, sigma ligands induce a rise in intracellular calcium levels (12). The pharmacological profile of all these effects indicates mediation by sigma-2 receptors. It has also been shown that exposure of tumor cells to sigma agonists results in inhibition of proliferation (13). Here we demonstrate that the mode of sigma-2 agonist-induced cell death is displays features consistent with apoptosis. Mutations in the tumor suppressor gene, p53, are the most frequently observed genetic aberrations in tumors, occurring in up to 50% of some tumor types. In tumor cells with p53 mutations, a diminished response to agents that induce apoptosis has been observed (14, 15), and these tumors may be clinically resistant to anti-neoplastic drugs that produce DNA-damage (16).

We show here that unlike DNA-damaging agents, sigma-2 receptor agonists exhibit similar potency in tumors with wild-type or mutant p53. The mechanism of sigma receptor-mediated apoptosis differs from that of agents that trigger DNA-damage, based on observations with inhibitors of caspases. The involvement of distinct apoptotic pathways is further supported by the ability of sigma agonists to potentiate the cytotoxicity of DNA-damaging antineoplastics in various tumor cell lines.

METHODS

I. Cell Culture

Human breast tumor cell lines (MCF-7, T47D, SKBr3, and MCF-7/Adr-) were cultured in DMEM containing 3.7g/liter Na_2HCO_3, fetal bovine serum (10%), and insulin (10 mg/liter). For cytotoxicity assays, cells were transferred to DMEM + Ham's nutrient mixture F-12 (without phenol red), with 1.2 g/liter Na_2HCO_3. Cells were seeded at 100,000 cells/well. Human SK-N-SH neuroblastoma and DU-145 prostate carcinoma cells were cultured in DMEM, supplemented with fetal bovine serum (10%).

II. Cytotoxicity assay

Cell death was assessed by release of lactate dehydrogenase (LDH) into the culture medium using the CytoTox 96 kit from Promega (Madison, WI). Method was as specified by manufacturer, with minor modifications. Cells were plated and cultured in 24-well plates for 1 to 2 days prior to experiment. Cells were treated in the plates under the specified drug conditions. Following treatment, the medium was transferred to centrifuge tubes and centrifuged to remove cell debris. Supernatant (50 µl) was transferred to 96-well plate, to which was added substrate mix in assay buffer, followed by stop solution after a 30 min incubation. Formation of formazan was monitored at 490 nM in a plate reader. Values are expressed relative to total lysis controls (Triton X-100 used to determine value for 100% cell kill) and no drug-controls. Doxorubicin and caspase Inhibitors (Z-VAD-FMK, Y-VAD-CHO, DEVD-CHO) were obtained from Calbiochem (San Diego, CA) and actinomycin-D from Sigma (St. Louis, MO).

III. Detection of Apoptosis

A. Annexin V binding

The early stages of apoptosis are characterized by translocation of phosphatidyl serine (PS) from the inner surface of the plasma membrane to the outer surface of the membrane. Externalized PS can then be detected using annexin V, a protein with high affinity for PS. This was carried out using the ApoAlert Annexin V Apoptosis Kit (Clontech, Palo Alto, CA) according to manufacturers specifications. After incubation with sigma ligands at the concentrations and times specified, live cells (without fixing) were incubated with annexin V-FITC and propidium iodide, and were investigated using fluorescence microscopy. Green staining shows externalized PS, while orange color revealed damage of cell membrane and penetration of propidium iodide to the cell interior, which occurs at the later stages of the process. Filters used: excitation 450 nm, barrier 535 nm.

B. TUNEL method

DNA fragmentation occurring during apoptosis can be detected by incorporating fluorescein-12-dUTP at the 3'-OH DNA ends using the enzyme, terminal deoxynucleotidyl transferase (TdT). TUNEL (TdT-mediated dUTP Nick-End Labeling) was performed using the Apoptosis Detection System, Fluorescein Kit (Promega, Madison, WI) according to manufacturers specification. After treatment with sigma ligands at the concentrations and times specified, cells were fixed with 4% formaldehyde, permeabilized with Triton X-100, and incubated with fluorescein-12-dUTP and terminal deoxynucleotidyl transferase and then with propidium iodide. Propidium iodide stains the DNA in both apoptotic and non-apoptotic cells with orange-red color. Fluorescein produces yellow-green fluorescence, indicating fragmented DNA within the nucleus of apoptotic cells. Filters used: excitation 450 - 490 nm, barrier 520 nm.

IV. Sigma Receptor Ligands

Haloperidol and reduced haloperidol are subtype non-selective sigma ligands (17) and were obtained from Research Biochemicals Inc. (Natick, MA). The 5-phenylmorphans CB-64D and CB-184 are sigma-2 subtype-selective agonists (18), synthesized in the Laboratory of Medicinal Chemistry/NIDDK (Dr. C. Bertha). CB-64D: (+)-1R,5R-*E*-8-benzylidene-5-(3-hydroxyphenyl)-2-

methylmorphan-7-one; CB-184: (+)-1R,5R-*E*-8-(3,4-dichlorobenzylidene)-5-(3-hydroxyphenyl)-2-methylmorphan-7-one

Results

Sigma Ligands Induce Apoptosis in Various Tumor Cell Lines.

The cell lines examined in this study are presented in table 1 along with the p53 genotype. The presence of p53 mutations renders some of these strains resistant to certain anti-neoplastic agents. Figure 1 shows that the MCF-7/Adr- displays diminished sensitivity to adriamycin compared to MCF-7 cells with WT p53. Various lines of cultured cells were incubated with sigma ligands and anti-neoplastic agents and the cells were assayed by the TUNEL method to assess drug-induced effects and potential mechanisms. Sigma ligands including CB64D, haloperidol, reduced haloperidol and antineoplastic agents such as doxorubicin, produced positive results in the TUNEL assay in MCF-7 and T47D cells (plate 1). The sigma-2 subtype selective ligand, CB64D, at a concentration of 100 uM produced extensive apoptotic changes in MCF-7 by 48 hours. In some experiments, up to 100% of cells visualized displayed apoptotic nuclei. The subtype non-selective sigma ligands, haloperidol and reduced haloperidol produced apoptotic nuclei, but less extensively than the more potent CB64D. Similarly, apoptosis was induced by doxorubicin and other antineoplastic drugs (actinomycin D, cyclophosphamide) in T47D and SKBr3 cell lines.

To quantify cell death induced by these agents, cultured cells were incubated in the presence of various concentrations of sigma 2 selective ligands, CB64D and CB184, at different time intervals, and cytotoxicity quantified by the release of lactate dehydrogenase (LDH) into cell culture media. Figure 2 shows a concentration dependent effect of the compounds on cytotoxicity in two cell lines, SkBr3 and T47D (figure 2). The potency of these compounds in inducing cell death is compared for four different cell lines in table 2. CB184 exhibits the greatest potency in all cell lines tested.

Involvement of Caspases in Cell Death

Caspases (cysteine aspartyl proteases) play a central role in the pathway of apoptosis by executing the apoptotic signals (19).

Both selective and non-selective inhibitors of caspases have been developed as biochemical tools to help disect the pathways by which an apoptotic signal is transmitted.

The ability of different caspase inhibitors to abrogate apoptosis induced by sigma ligands and some anti-neoplastic drugs was compared.

MCF-7 cells were treated with the sigma ligand CB64D (100 μM) in the presence or absence of YVAD-CHO (50 μM), an inhibitor of caspase 1 or ZVAD-FMK (50 μM) an inhibitor of all known caspases, for various time periods. Apoptosis was determined by the binding of Annexin V to the treated cells. The apoptotic changes occurring in the CB-64D treated cells were unaffected by co-treatment with caspase inhibitors (plate 2).

Actinomycin D has been shown to produce apoptosis by either inhibition of nucleic acid synthesis or by intercalation with DNA base pairs leading to the induction of p53 (20). MCF-7 cells were treated with varying concentrations of either CB184 or actinomycin D in the presence or absence of DEVD-CHO (50 μM), a specific inhibitor of caspase 3. Cytotoxicity was assessed by LDH release. DEVD-CHO abrogated actinomycin D cytotoxicity at its higher doses whereas in cells treated with CB184, there was no difference in the presence or absence of DEVD-CHO on cell killing (figure 3). These observations were consistent with the effects of caspase-inhibitors in the presence of the sigma-2 selective CB64D in the Annexin V binding experiments above. Doxorubicin induced a dose-dependent increase in LDH-release, where the effect of the lower doses was abolished by Z-VAD-FMK (figure 3).

Sigma Agonists Potentiate Antineoplastic Agents

The combination of the sigma 2 agonist with anti-neoplasic agents, at concentrations of the individual agents producing modest to no effects on cell killing, resulted in substantial cytotoxicity.

Clear synergistic effects were observed at 24 hours when CB184 (1 μM) was combined with various doses of doxorubicin. This effect was observed in MCF-7 cells (figure 4) and also in MCF-7/Adr-, but less pronounced. Cytotoxicity was also potentiated with the combination of CB184 and actinomycin D (10μg/ml) at 24 hours, and at lower concentrations of actinomycin D (1μg/ml) displayed the same response but at a later time point (figure 4).

To determine if clinically available sigma agonists displayed similar effects as CB184, the non-selective sigma agonists haloperidol and pentazocine (the racemic mixture) were combined with doxorubicin at various concentrations. In MCF-7 /Adr- cell, haloperidol (25 μM) markedly potentiated doxorubicin, and to a lesser extent pentazocine (35 μM) potentiated the higher doses of doxorubicin. Little or no potentiation was observed by these agents when combined with doxorubicin in MCF-7 cells.

Discussion

We have shown the ability of sigma-2 agonists to produce apoptosis and cell death by mechanisms that appear to be independent of p53 and independent of caspases.

Mutations in p53 often confer resistance to apoptosis and resistance to DNA-damaging agents that induce apoptosis, although some studies show increased sensitivity in p53 mutants (see 21). The p53 mutant cell lines that we have examined displayed chemoresistance to certain agents (see figure 1) and similar observations have been previously reported (MCF/Adr- to doxorubicin, 22 and TNF-α; 23; SKBr3 to several agents; 21).

We have used LDH release as a method to quantify cell death, but this method doesn't distinquish apoptotic from necrotic cell death. At the appropriate sigma ligand concentration and duration, up to 100% cell killing can be observed with our potent selective agents CB64D and CB184. Similarly, under the same conditions, we can observe 100% of cells undergoing apoptosis when analyzed both by the TUNEL assay and Annexin V binding, though the optimal duration may

vary between the two assays. These observations are suggestive that the mode of cell death is apoptotic.

Caspases are a family of cysteine-aspartyl proteases that are the executioners of apoptotic signals from diverse stimuli including receptor activation (eg. Fas ligand, TNFα), DNA-damaging agents (eg. doxorubicin, alkylating agents), hypoxia, growth factor deprivation or ionizing radiation. The targets of caspases include a vast array of cytoskeletal proteins, cell cycle regulatory proteins and nuclear matrix proteins such that the proteolytic cleavage of these targets are consistent with the morphological and biochemical alterations characteristic of apoptosis. Most apoptotic signals initiate a cascade of sequential caspase activation (19), although caspase independent pathways have also been described (some discussed below). In order to further characterize the apoptotic effect observed here, we compared the effects of the caspase inhibitors on the actions of sigma 2 agonists in the same experiments with doxorubicin and actinomycin D. Suprisingly, inhibitors of caspases had no effect on cytotoxicity of the sigma 2 agonists, whereas the response to the anti-neoplastics was prevented, as observed by others (24, 25).

Whether apoptosis can proceed in the absence of caspase activation is an important question. There are reports of several different pathways by which compounds can produce caspase-independent apoptosis, and these studies may aid in elucidating pathways involved in sigma 2 -mediated cell death. Laethem et al. (1998) observed that blockade of caspase 3 (CPP32) had no effect on various morphological changes characteristic of apoptosis induced by ceramide, but abolished DNA-degradation. The protein calcineurin is activated to induce apoptosis by elevations and cytosolic calcium, which subsequently leads to activation of the pro-apoptotic protein BAD. These effects occur independent of caspases (27; 28). Elevations in cytosolic calcium are observed during chronic exposure to sigma 2 ligands and appear to be involved in the apoptotic signal (29). Bax-induced cell death may occur independently of ICE-proteases (30). Mitochondrial changes characteristic of apoptosis occurs in yeasts, which lack caspases (31). Bin1, a tumor

suppressor often lost in breast cancers, interacts with myc to trigger apoptosis independently of p53. Its action is insensentive to inhibition of caspases but can be abolished by certain serine-protease inhibitors. DNA-damaging agents do not alter Bin1 levels (32).

The observation that sigma-2 agonists are equipotent in killing cells with mutant and wild-type p53 genes and can potentiate anti-neoplastic drug effects in breast tumor cells has tremendous implications for clinical practice. The utility of potent sigma ligands for treating tumors could be limited since many tissues normally express high-densities of the receptor (3) and chronic exposure to these agents may have deleterious effects. The ability of these compounds to potentiate anti-neoplastics means that lower doses of the sigma compounds are required to enhance the effects of drugs which already have some limited selectivity for tumors. Furthermore, the sigma-2 agonist was able to potentiate at a dose which alone was not cytotoxic. This phenomenon may also result in reversal of drug resistance in tumors at concentrations of the drug that reduce the very severe adverse effects.

SKBr3 cells overexpress erbB-2 protein and also possess a p53 mutation that give these cells a growth advantage and reduced estrogen dependence (32). In patients with tumors that overexpress erbB-2 coupled with p53 mutations, improved responses are obtained from the high-dose CAF regimen. When different regimens were compared in patients with tumors possessing these biologic markers, the greatest benefit was obtained from Adriamycin-containing regimens (33). These observations suggest a tremendous potential benefit in sigma agonists to optimize the inclusion of adriamycin at doses that minimize the toxicities of adriamycin. The clinically available sigma agonists, also display the potentiation phenomena. The butyrophenones, droperidol and haloperidol, have efficacy against anti-neoplastic-induced emesis of moderate severity, although haloperidol is seldom used for this indication. Racemic pentazicine (Talwin) can be used in the management of moderate pain due to metastatic tumors. Therefore, these agents that can potentiate anti-neoplastic activity have other actions useful in cancer patients. The doses of haloperidol and

pentazocine that potentiate doxorubicin are higher than the standard doses. But because these drugs reduce the time to observe anti-neoplastic drug killing, they may be co-administered with doxorubicin for a limited duration which may limit toxicity. Pentazocine when dosed at 1.2 mg/kg administered intravenously, may produce dysphoric/psychomimetic effects as well as cardiovascular adverse effects that can be antagonized by naloxone.

The MCF-7/Adr- cells with mutant p53 have also been shown to over-express the MDR gene product, p-glycoprotein (22). P-glycoprotein enhances the efflux of hydrophobic compounds that are often toxic to cells. Doxorubicin is a substrate for MDR and over-expression of MDR results in phenotypic resistance to doxorubicin, even in the presence of wild-type p53. Sigma 2 agonists have been shown to reduce the expression of the MDR gene (34). This is not likely to be the predominant mechanism for the potentiation we observed, since it occurs in MCF-7 cells that are sensitive to doxorubicin, and do not over-express MDR.

In summary, sigma-2 receptor agonists induced apoptosis in various breast tumor cell lines in a manner apparently independent of both p53 and caspase activation. Also, sigma-2 agonists at doses that which were not cytotoxic, potentiated the action of DNA-damaging agents. This suggests that sigma-2 receptors utilize an apoptotic pathway distinct from that utilized by DNA-damaging agents and other apoptotic stimuli. Sigma-2 receptors may represent novel targets for the development of antineoplastic agents.

Breast Cell Lines Studied

MCF-7	**breast epithelium**	**wild-type p53 protein**
MCF-7/Adr-*	**p53 exon 5 mutation; 21 bp deletion (126-133)**	
SKBr3*	**mutation at codon 175 of p53 gene**	
T47D*	**mutation at codon 194 of p53 gene**	

Fig. 1. Comparison of Sensitivity to Doxorubicin of MCF-7 Cells and MCF-7/Adr-Cells.

Cells were incubated in the presence of various concentration of doxorubicin . Cytotoxicity was determined by release of lactate dehydrogenase into culture media and expressed as percentage of total cell kill as described in *Methods*. The above figure is representative for a 48 hr. time point. Each data point represents the mean and standard error of duplicate samplings from two culture wells at each concentration (4 samplings).

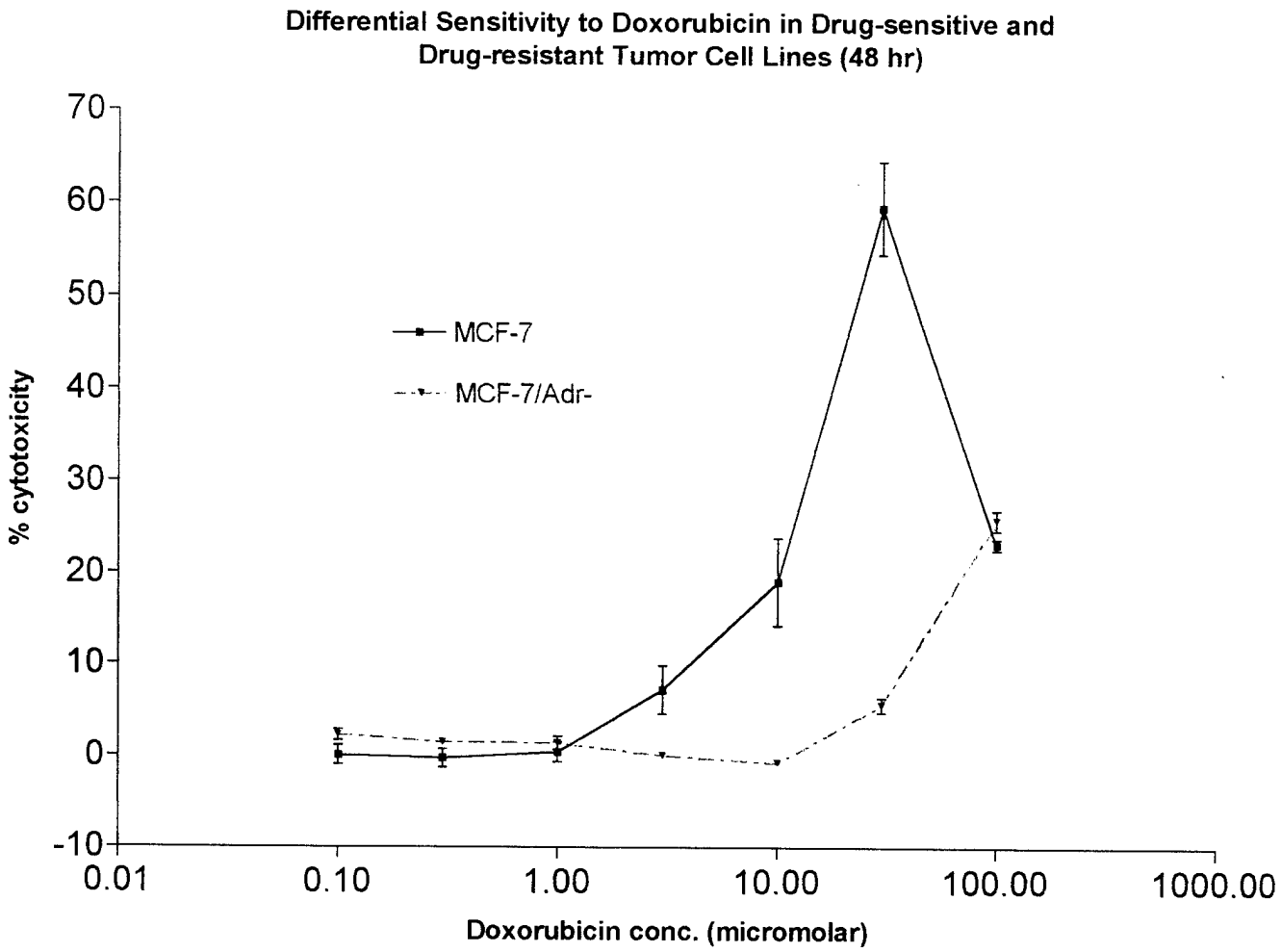
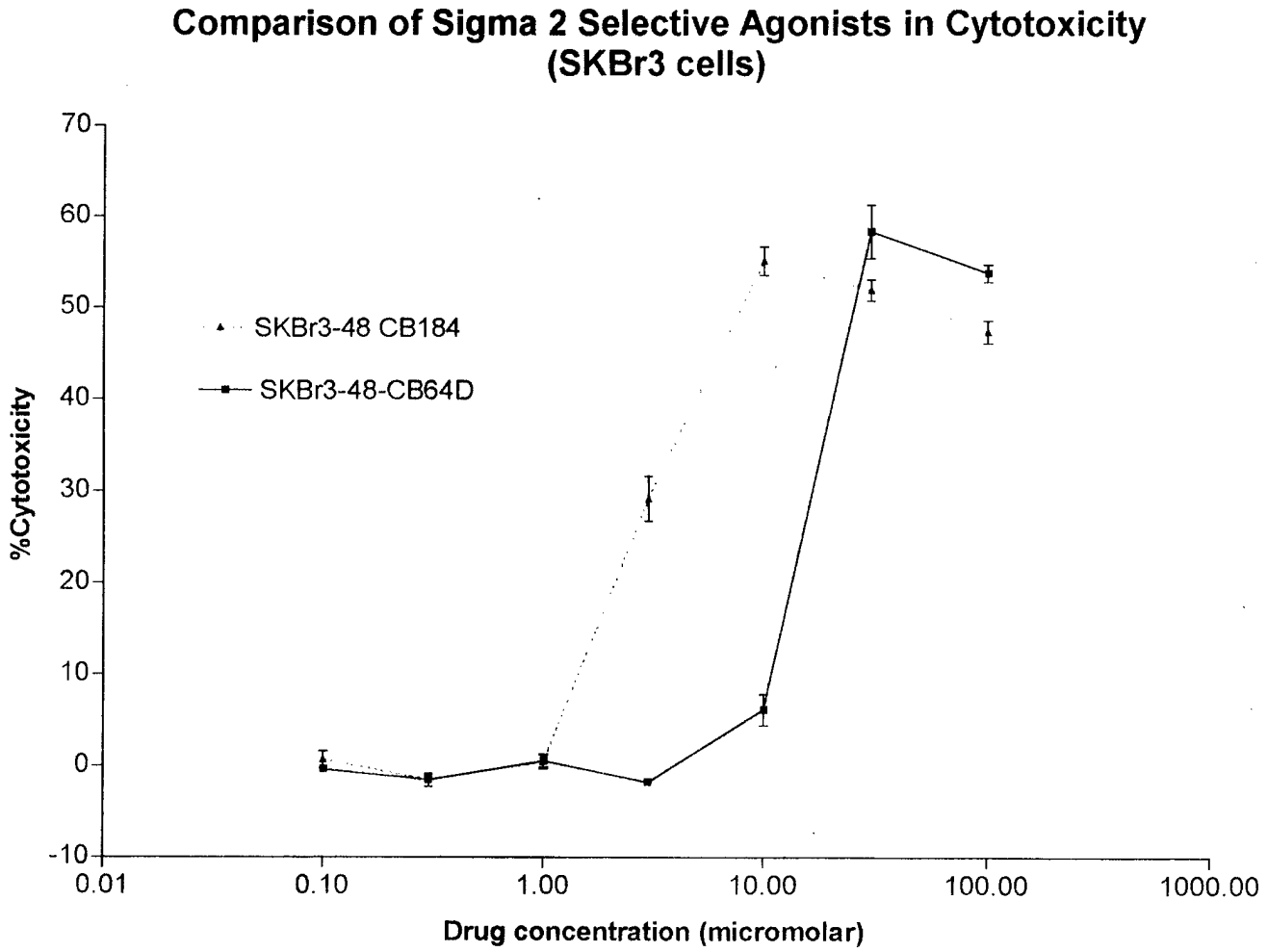

Differential Sensitivity to Doxorubicin in Drug-sensitive and Drug-resistant Tumor Cell Lines (48 hr)
% cytotoxicity
70
60
50
40
30
20
10
0
-10
MCF-7
MCF-7/Adr-
0.01
0.10
1.00
10.00
100.00
1000.00
Doxorubicin conc. (micromolar)

Plate 1. Sigma Agonists and DNA-Damaging Antineoplastic Agents Induce Apoptosis in Various Tumor Cell Lines.

MCF-7 cells or T47D cells were incubated with the DNA-damaging agent, doxorubicin or with the sigma receptor agonists CB-64D, haloperidol, and reduced haloperidol. Cells were assayed for apoptosis by the TUNEL method, and photographed by fluorescence microscopy with a filter at 40x magnification. Green or yellowish-green cells are positive for DNA-fragmentation consistent with apoptosis. Red or orange-red cells are non-apoptotic propidium iodide staining nuclei. Compounds were used at a concentration of 100μM.

Fig. 2. Concentration-Dependent Effect of Sigma-2 Agonists on Cell Killing in Different Tumor Cell Lines.

SKBr3 cells (panel A) and T47D (panel B) were incubated in the presence of various concentrations of the sigma-2 agonists CB-64D and CB-184, as described in *Methods* at the designated times. Cytotoxicity was determined by measuring the release of lactate dehydrogenase into culture media and expressed as percentage of total cell kill. Each data point represents the mean and standard error of duplicate samplings from two culture wells at each concentration (4 samplings). Each figure is representative of 3 or 4 experiments. Experiments were also performed using MCF-7 and MCF-7/Adr- cell lines (data not shown, but see Table 2).


Comparison of Sigma 2 Selective Agonists in Cytotoxicity
(SKBr3 cells)
%Cytotoxicity
70
60
50
40
30
20
10
0
-10
SKBr3-48 CB184
SKBr3-48-CB64D
0.01
0.10
1.00
10.00
100.00
1000.00
Drug concentration (micromolar)

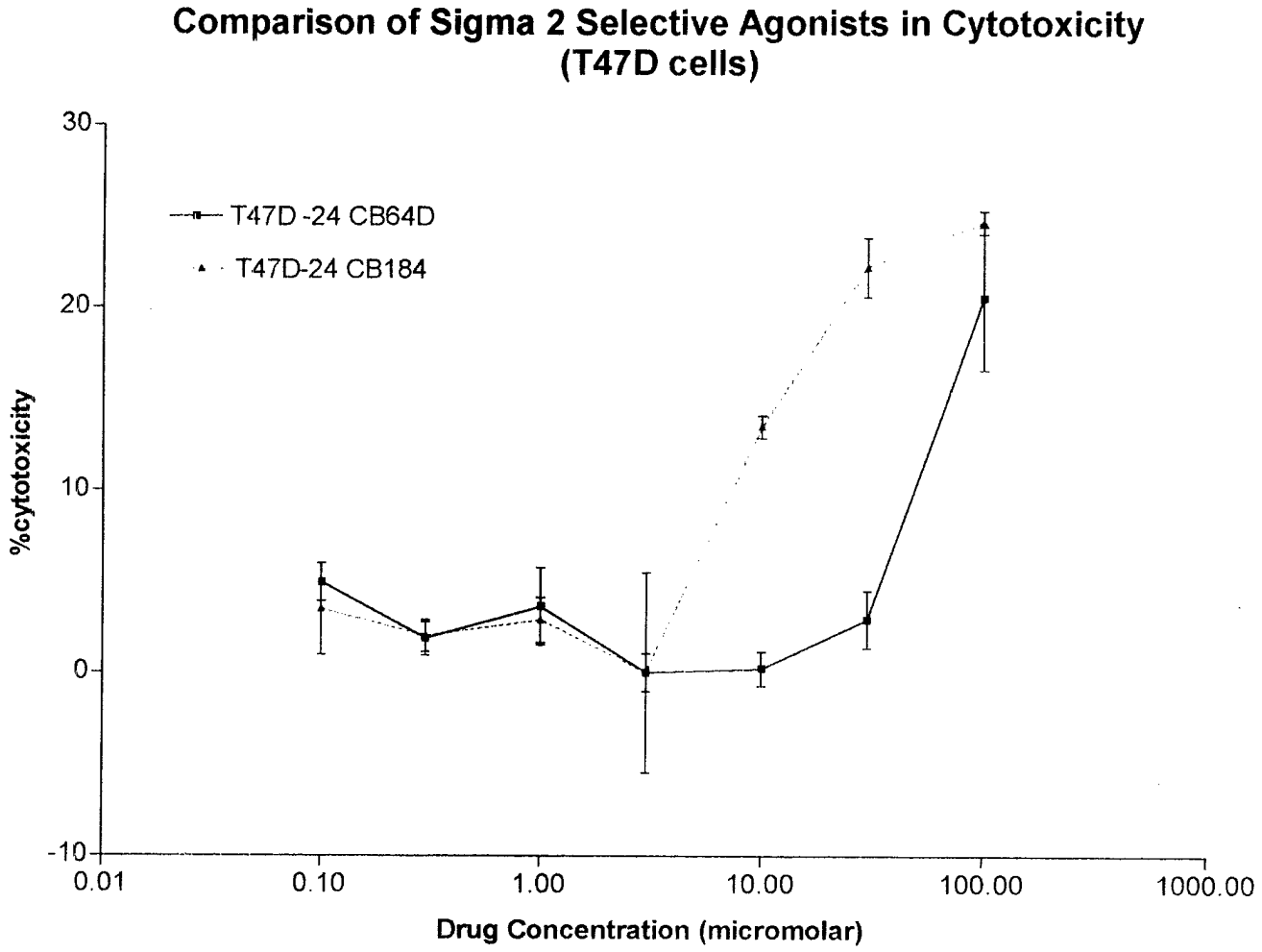

Comparison of Sigma 2 Selective Agonists in Cytotoxicity
(T47D cells)
T47D -24 CB64D
T47D-24 CB184
%cytotoxicity
30
20
10
0
-10
0.01
0.10
1.00
10.00
100.00
1000.00
Drug Concentration (micromolar)

Table2. EC50 (μM) for cytotoxic response to Sigma 2 Agonists in Breast tumor cell lines

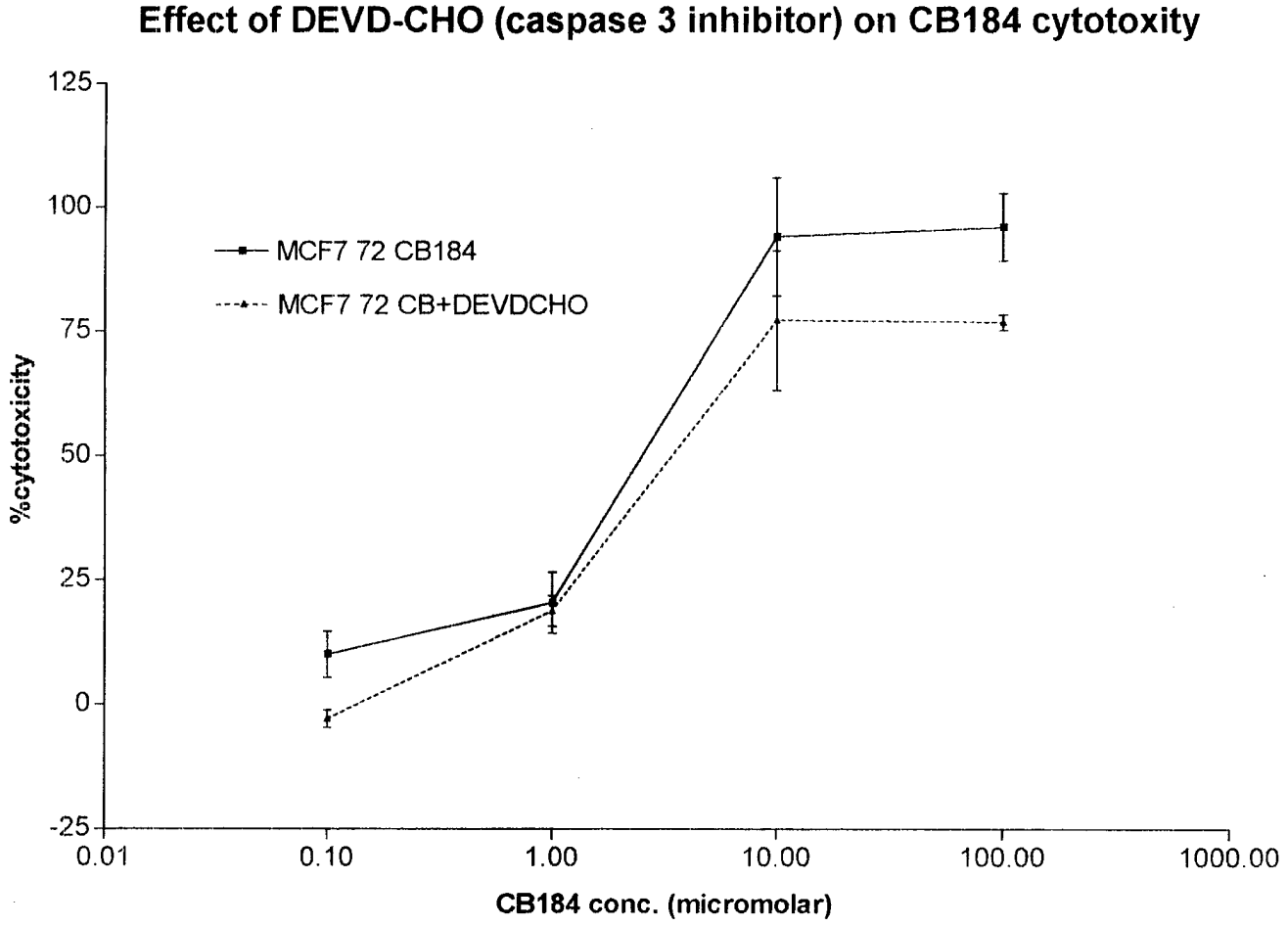
Cell lines	CB64D	CB184
MCF-7	36.33 ± 2.2	4.3 ± 1.8
MCF-7/Adr-	12.4 ± 0.06	5.8 ± 0.25
SkBr3	40.24 ± 12.1	5.0 ± 1.81
T47D	73.01 ± 6.6	9.0 ± 5.45

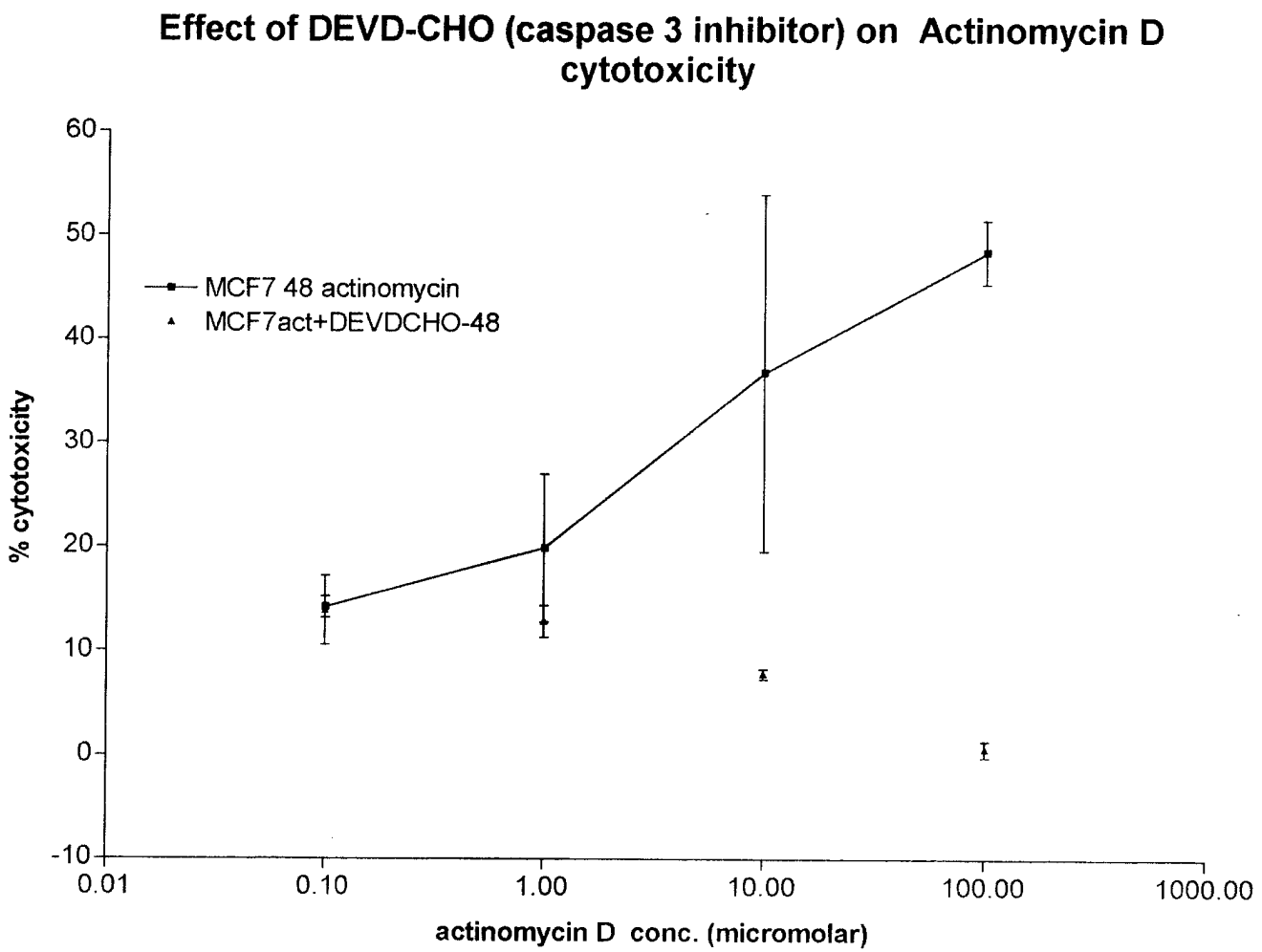
Plate 2. Sigma-2 Agonists in the Induction of Apoptosis: Effect of Caspase Inhibitors.

MCF-7 cells were grown as described in *Methods* and incubated with drugs in the presence or absence of the non-selective caspase inhibitor Z-VAD-FMK or the selective caspase-1 inhibitor Y-VAD-CHO. Apoptosis was determined using Annexin V binding. Treatments were as follows: control, 48 hrs; CB-64D 100 μM; CB-64D + Z-VAD-FMK; CB-64D Y-VAD-CHO.

Fig. 3. Effect of Caspase Inhibitors on Drug-Induced Cytotoxicity in MCF-7 Cells: Sigma-2 Agonists and Antineoplastic Drugs have Different Mechanisms of Action.

MCF-7 cells were treated with various concentrations of CB-184, doxorubicin or actinomycin-D, either with or without DEVD-CHO (50 μM) or Z-VAD-FMK (50 μM) for the designated times. Cytotoxicity was determined by measuring the release of lactate dehydrogenase into culture media and expressed as a percentage of total cell kill. Each data point represents the mean and standard error of duplicate samplings from two culture wells for each treatment group (4 samplings). Each figure is representative of 3 experiments (* $p < 0.01$, ** $p < 0.001$ by Student's two-tailed T-test).


Effect of DEVD-CHO (caspase 3 inhibitor) on CB184 cytotoxity
125
100
75
50
25
0
-25
%cytotoxicity
MCF7 72 CB184
MCF7 72 CB+DEVDCHO
0.01
0.10
1.00
10.00
100.00
1000.00
CB184 conc. (micromolar)


Effect of DEVD-CHO (caspase 3 inhibitor) on Actinomycin D cytotoxicity
MCF7 48 actinomycin
MCF7act+DEVDCHO-48
% cytotoxicity
60
50
40
30
20
10
0
-10
0.01
0.10
1.00
10.00
100.00
1000.00
actinomycin D conc. (micromolar)

dox-zvad.pzm:Graph-1 - Wed Jun 09 14:22:33 1999

Effect of Caspase Inhibition on Doxorubicin-induced Cytotoxity in MCF-7 cells(48 hr)

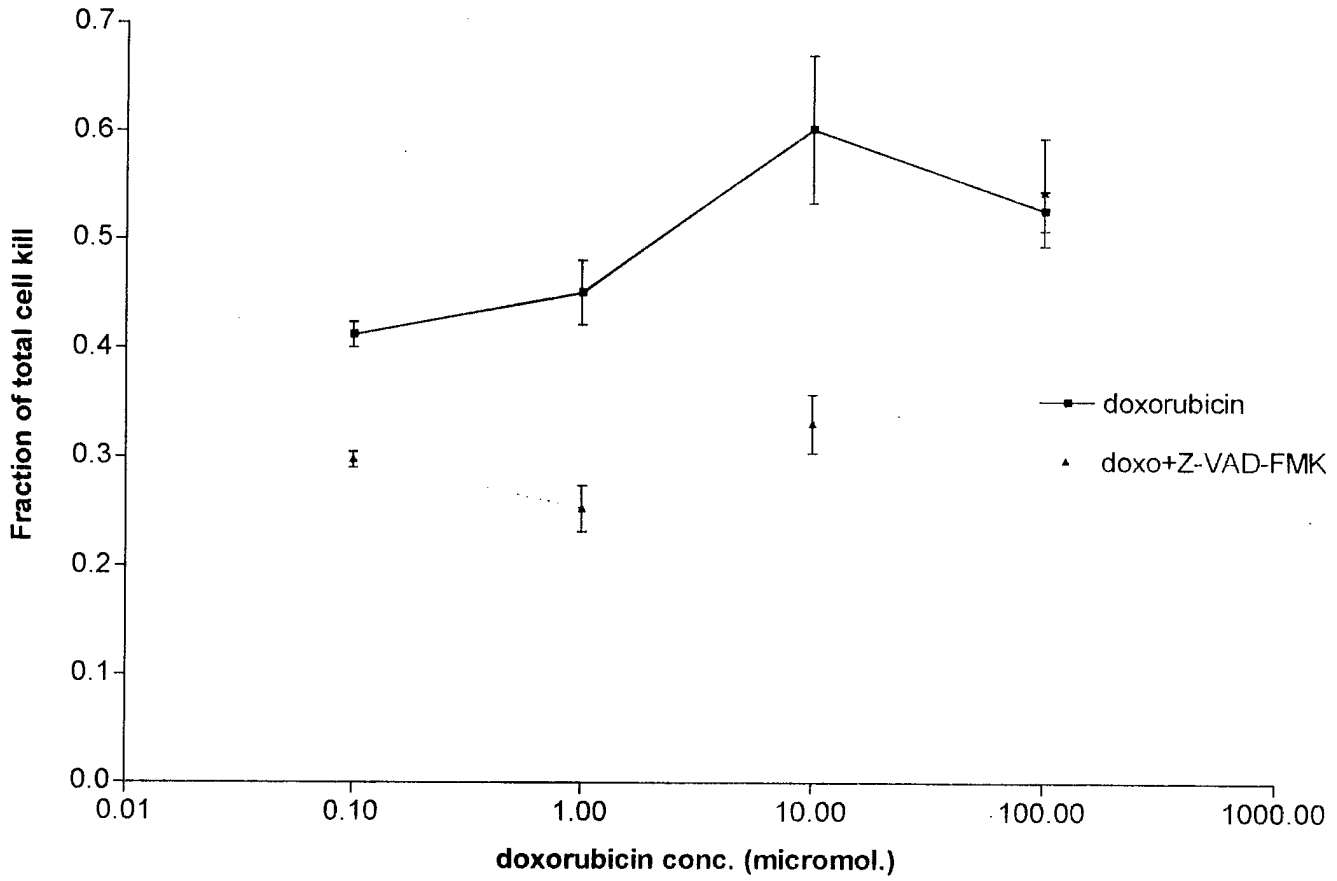
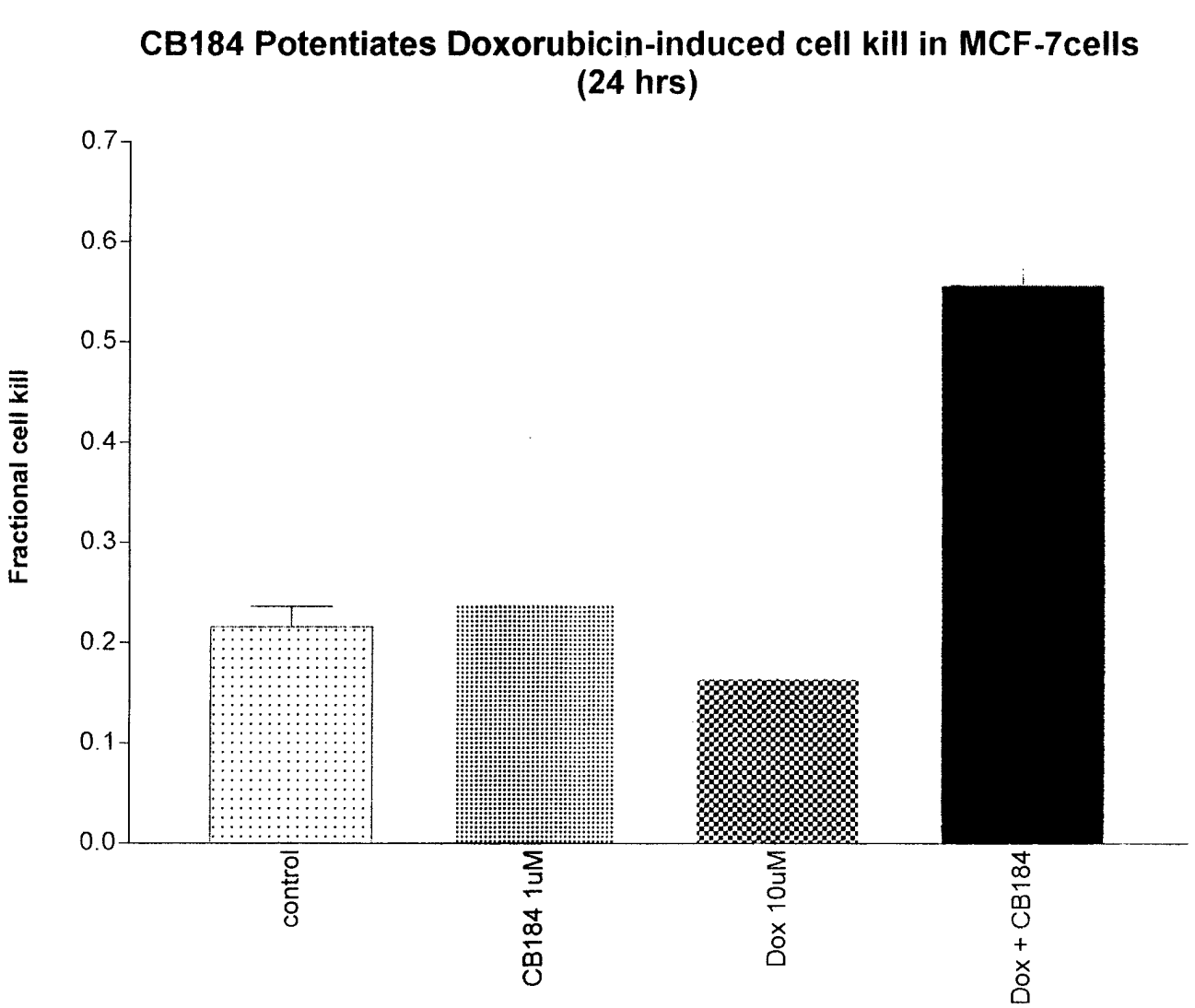
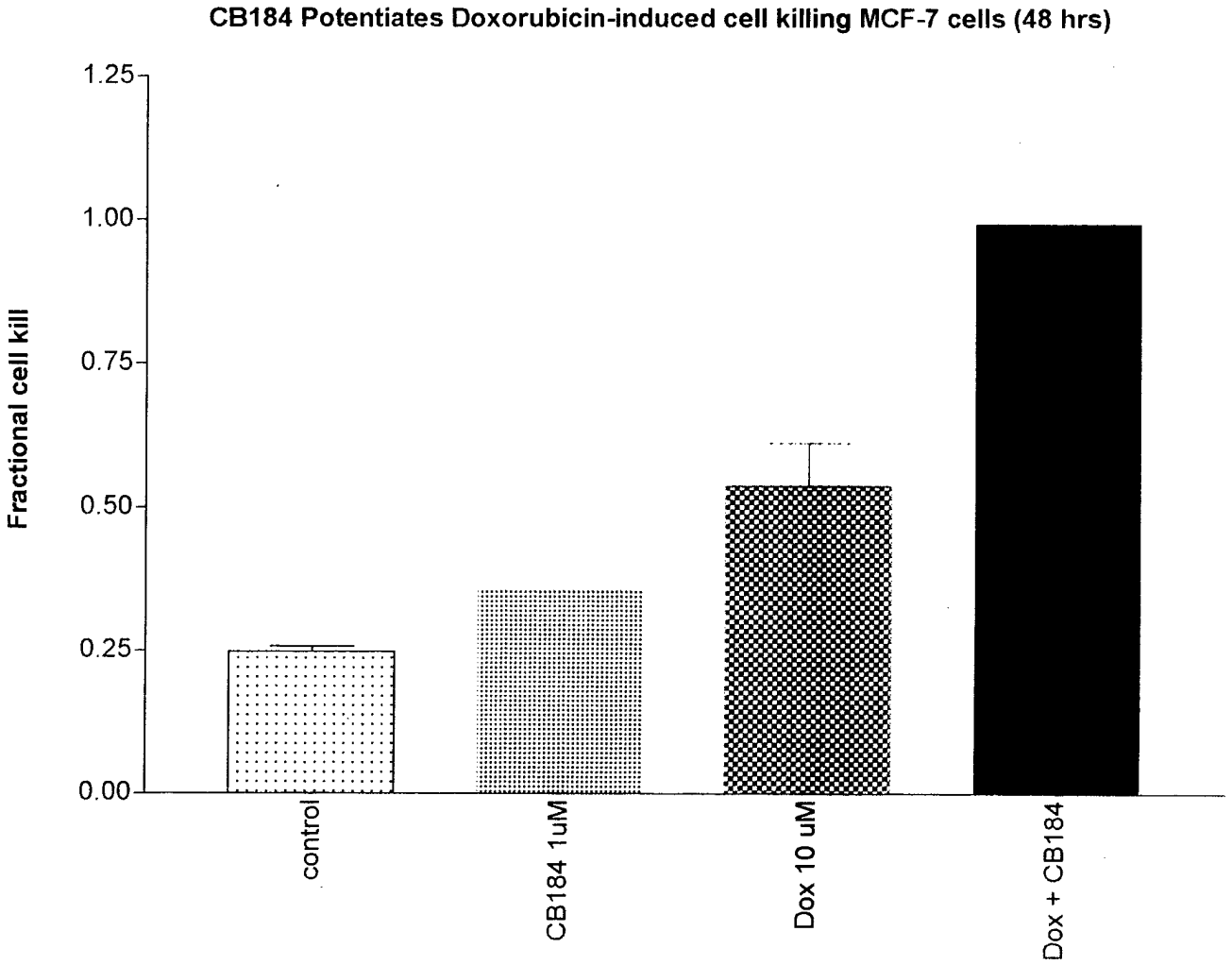
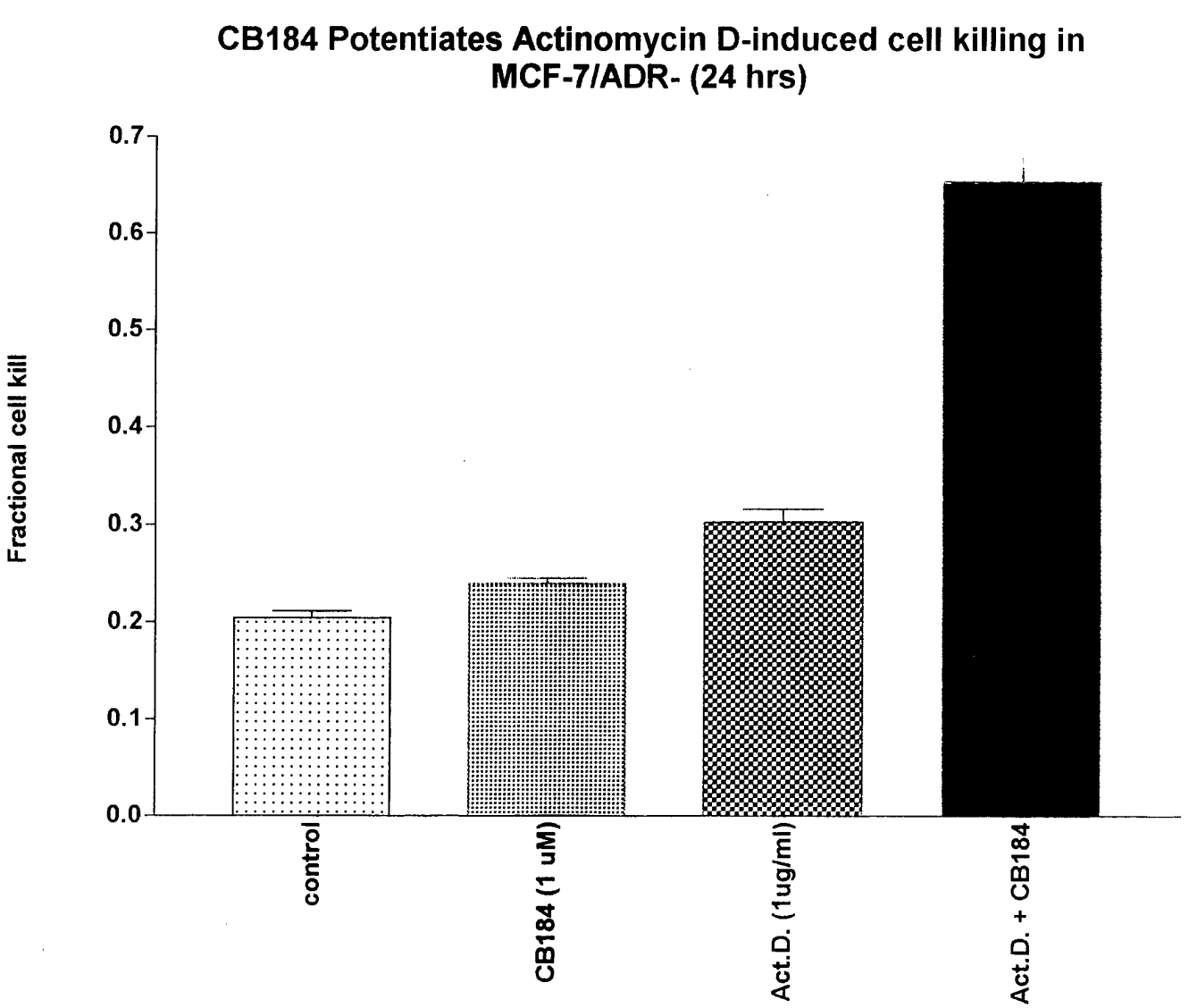


Fig. 4. Combining Antineoplastic Drugs with a Sigma Agonist Potentiates Cytotoxicity.
CB-184 (1 μM) was combined with doxorubicin (10 μM) in MCF-7 cells (24 hr, panel A; 48 hr, panel B), or with actinomycin-D in MCF-7/Adr$^-$ (1 μg/ml at 24 hr, panel C; 0.1 μg/ml at 48 hr, panel D). Cytotoxicity was measured by lactate dehydrogenase release into culture media. Each bar represents the mean value and standard error of duplicate samplings from two culture wells for each treatment group (4 samplings). These figures are representative of 2 – 3 experiments (* $p < 0.001$ comparing combined groups with each of the single drug treatment groups).


CB184 Potentiates Doxorubicin-induced cell kill in MCF-7cells
(24 hrs)
Fractional cell kill
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
CB184 1uM
Dox 10uM
Dox + CB184


CB184 Potentiates Doxorubicin-induced cell killing MCF-7 cells (48 hrs)
Fractional cell kill
1.25
1.00
0.75
0.50
0.25
0.00
control
CB184 1uM
Dox 10 uM
Dox + CB184


CB184 Potentiates Actinomycin D-induced cell killing in MCF-7/ADR- (24 hrs)
Fractional cell kill
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
CB184 (1 uM)
Act.D. (1ug/ml)
Act.D. + CB184

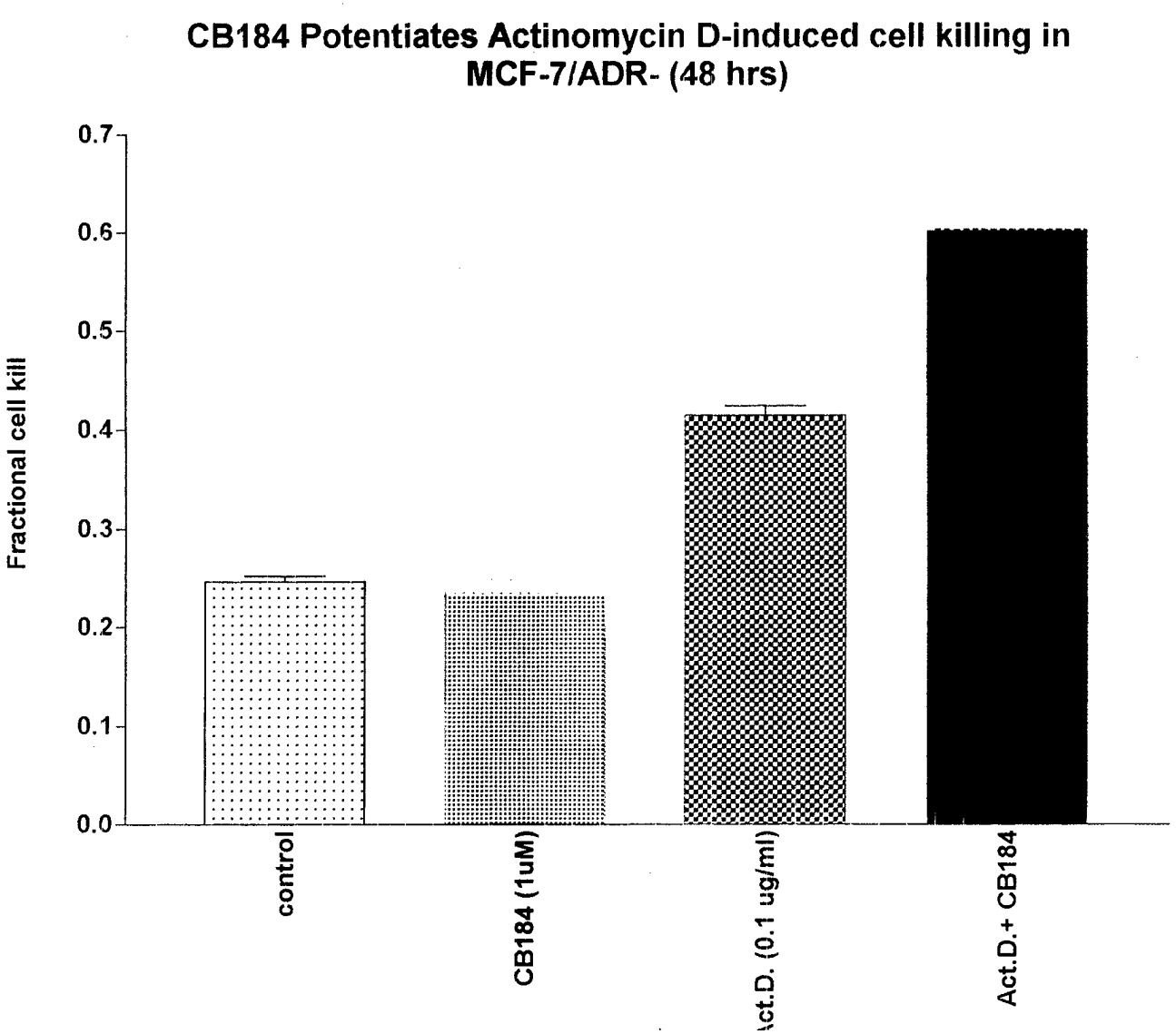
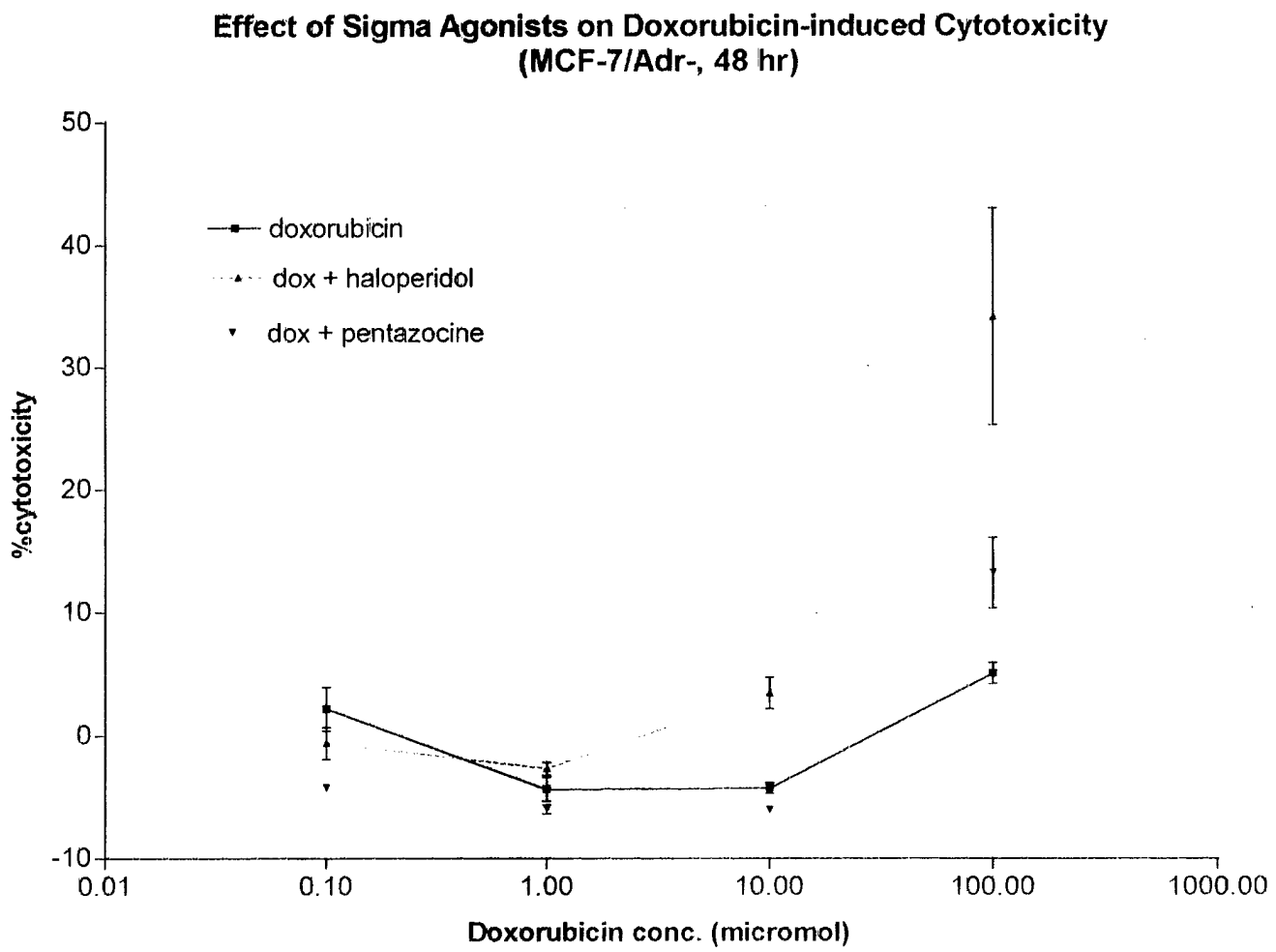

CB184 Potentiates Actinomycin D-induced cell killing in MCF-7/ADR- (48 hrs)
Fractional cell kill
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
CB184 (1uM)
ct.D. (0.1 ug/ml)
Act.D.+ CB184

Fig. 5. Effect of Sigma Agonists on Doxorubicin-induced Cytotoxicity in MCF-7/Adr- Tumor Cells.
MCF-7/Adr- cells were incubated with various concentrations of doxorubicin alone or in the presence haloperidol (25 μM) or pentazocine (35 μM). . Cytotoxicity was determined by measuring the release of lactate dehydrogenase into culture media and expressed as a percentage of total cell kill. Each data point represents the mean and standard error of duplicate samplings from two culture wells for each treatment group (4 samplings). This figure is representative of 3 experiments.


Effect of Sigma Agonists on Doxorubicin-induced Cytotoxicity
(MCF-7/Adr-, 48 hr)
doxorubicin
dox + haloperidol
dox + pentazocine
%cytotoxicity
50
40
30
20
10
0
-10
0.01
0.10
1.00
10.00
100.00
1000.00
Doxorubicin conc. (micromol)

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REFERENCES

1. Walker, J.M., Bowen, W.D., Walker, F.O., Matsumoto, R.R., de Costa, B.R., and Rice, K.C. Sigma receptors: Biology and function. *Pharmacological Reviews* 42: 355-402, 1990.

2. Su, T.-P., London, E.D., and Jeffe, J.H. Steroid binding at sigma receptors suggests a link between endocrine, nervous, and immune systems. *Science* 240: 219-221, 1988.

3. Hellewell, S.B. and Bowen, W.D. A sigma-like binding site in rat pheochromocytoma (PC12) cells: Decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that in guinea pig brain. *Brain Res.* 527: 244-253, 1990.

4. Quirion, R., Bowen, W.D., Itzhak, Y., Junien, J.L., Musacchio, J.M., Rothman, R.B., Su, T.-P., Tam,. S.W., and Taylor, D.P. A proposal for the classification of sigma binding sites. *Trends Pharmacol. Sci.* 13: 85-86, 1992.

5. Hellewell, S.B., Bruce, A., Feinstein, G., Orringer, J., Williams, W., and Bowen, W.D. Rat liver and kidney contain high densities of sigma-1 and sigma-2 receptors: Characterization by ligand binding and photoaffinity labeling. *Eur. J. Pharmacol. - Mol. Pharmacol. Sect.* 268: 9-18, 1994.

6. Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.-G., Striessnig, J., Kempner, E., and Glossmann, H. Purification, molecular cloning, and expression of the mammalian sigma$_1$-binding site. *Proc. Natl. Acad. Sci. USA* 93: 8072-8077, 1996.

7. Vilner, B.J., John, C.S., and Bowen, W.D. Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Res.* 55: 408-413, 1995.

8. Mach, R.H., Smith, C.R., al-Nabulsi, I., Whirrett, B.R., Childers, S.R., and Wheeler, K.T. Sigma-2 receptors as potential biomarkers of proliferation in breast cancer. *Can. Res.* 57: 156-161, 1997.

9. John, C.S., Vilner, B.J., Schwartz, A.M., and Bowen, W.D. Characterization of sigma receptor binding sites in human biopsied solid breast tumors. *J. Nucl. Med.* 37: 267P, 1996.

10. John, C.S., Bowen, W.D., Saga, T., Kinuya, S., Vilner, B.J., Baumgold, J., Paik, C.H., Reba, R.C., Neumann, R.D., Varma, V.M., and McAfee, J.G. A malignant melanoma imaging agent: Synthesis, characterization, *in vitro* binding and biodistribution of iodine-125-(2-piperidinylaminoethyl)4-iodobenzamide. *J. Nucl. Med.* 34: 2169-2175, 1993.

11. Vilner, B.J., de Costa, B.R., and Bowen, W.D. Cytotoxic effects of sigma ligands: Sigma receptor-mediated alterations in cellular morphology and viability. *J. Neurosci.* 15: 117-134, 1995.

12. Vilner, B.J. and Bowen, W.D. Dual modulation of cellular calcium by sigma receptor ligands: Release from intracellular stores and blockade of voltage-dependent influx. *Soc. Neurosci. Abstr.* 21: 1608, #631.3., 1995.

13. Brent, P.J. and Pang, G.T. Sigma binding site ligands inhibit cell proliferation in mammary and colon carcinoma cell lines and melanoma cells in culture. *Eur. J. Pharmacol.* 278: 151–160, 1995.

14. Ryan, K.M. and Vousden, K.H. Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Mol. Cell. Biol. 18: 3692-3698, 1998.

15. Lomax, M.E., Barnes, D.M., Hupp, T.R., Picksley, S.M., and Camplejohn, R.S. Characterization of p53 oligomerization domain mutations isolated from Li-Fraumeni and Li-Fraumeni like family members. Oncogene 17: 643-649, 1998.

16. Wallace-Brodeur, R.R. and Lowe, S.W. Clinical implications of p53 mutations. Cell. Mol. Life Sci. 55: 64-75, 1999.

17. Bowen, W.D., Moses, E.L., Tolentino, P.J., and Walker, J.M. Metabolites of haloperidol display preferential activity at sigma receptors compared to dopamine D-2 receptors. *Eur. J. Pharmacol.* 177: 111-118, 1990.

18. Bowen, W.D., Bertha, C.M., Vilner, B.J., and Rice, K.C. CB-64D and CB-184: Ligands with high sigma-2 receptor affinity and subtype selectivity. *Eur. J. Pharmacol.* 278: 257-260, 1995.

19. Cohen, GM Caspases;the executioners of Apoptosis. Biochemical Journal 1997, 326:1-16

20. Martin, SJ; Lennon, SV, Bonham, AM; Cotter, TG Induction of Apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthyesis. Journal of I mmunology 1990, 145:1859-1867

21. Blagosklonny, MV; El-Dierry WS Acute Overexpression of WT P53 facilitates anticancer drug-induced dearth of cancer and normal cells. International Journal of Cancer 1998, 75: 933-940

22. Ogretmen, B; Safa, AR. Expression of the mutated p53 tumor suppressor protein and its molecular and bichemical characterization in multidrug resistant MCF-7/Adt human breast cancer cells. Oncogene 1997, 14:499-506

23. Cai, Z,; Capoulade, C.; Moyret-Lalle, C. et al. Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with losss of p53 function. Oncogene 1997, 15:2817-2826

24. Martinez-Lorenzo, MJ; Gamen,S, Etxeberria, J et al. Resistance to Apoptosis Correlates with a Highly Proliferative Phenotype and Loss of FAS and CPP32 (Caspase-3) Expression in Human Leukemia Cells Int. J. Cancer (1998) 75: 473-81

25. Gamen, S, Anel, A., Lasierra, P. et al. Doxorubicin-induced Apoptosis in Human T-cell Leukemia is Mediated by Caspase-3 Activation in a FAS-Independent Way . FEBS Letters (1997) 417:360-4

26. Laethem, R.; Hannun, Y.; Jayadev, S. et al. Increases in neutral, Mg2+-dependent and acidic,

Mg2+-independent sphingomyelinase activities precede commitment to apoptosis and are not a consequence of caspase 3-like activity in Molt-4 cells in response to thymidylate synthase inhibition by GW1843. Blood 1998 91:4350-4360

27. Wang, HG; Pathan, N.; Ethell, IM. et al. Ca2+-induced apoptosis through calcineurin dephosporylation of BAD. Science 1999 284(5412):339-343

28. Yamamoto, AM; Eloy L.; Bach, J-F.; Garchon, H-J. N-terminus cleavage of bcl-2 by anovel cellular non-ICE cysteine proteinase. Leukemia 1998 12:1467-1472

29. Vilner, B; Bowen, W Modulation of Cellular calcium by Sigma Receptor Ligands: Release form Intra-cellular Stores in Human SK-N-SH Neuroblastoma Cells. submitted

30. Xiang, J.; Chao, DT.; Korsemeyer, SJ. BAX-induced cell death may not require interleukin 1β-converting enzyme-like proteases. Proceedings National Academy of Sciences 1996 93:14559-14563

31. Priault, M.; Chaudhuri, B.; Clow, A. et al. Investigation of bar-induced release of cytochrome c from yeast mitochondria-permeability of mitochondrial membranes, role of VDAC and ATP-requirement. 1999 260(3):684-691

32. Elliott, K; Ge, K; Prendergast, GC Loss of Myc-Bin1 Apoptosis Pathway in Cancer cells. Proceeding of the American Association of Cancer Research (1999) #2032

33. Liu, YL; Elashry, D; Chen, D. MCF-7 Breast-Cancer Cells Overexpressing Transfected C-ERBB-2 Have an In-Vitro Growth Advantage in Estrogen-depleted Conditions and Reduced Estrogen-dependence and Tamoxifen-sensitivity in-Vivo. Breast Cancer Research and Treatment (1995) 34: (2) 97-117

34. Thor AD; Berry DA; Budman DR et al. erb B-2, p53, and Efficacy of Adjuvant Therapy in Lymph Node-positive Breast Cancer. Int. J. Cancer (1998) 90: (18) 1346-60

35. Linn, SC; Honkoop, AH; Hoekman,K. et al. p53 and P-glyprotein are often co-expressed and are associated with poor prognosis in breast cancer. British Journal of Cancer 1996 74:63-68

36. Lyn-Cook, BD; Jin, B; Blann, E. et al. Sigma Receptor Ligands Modulate Expression of the Multi-drug Resistance Gene in Human and Rodent Brain Tumor Cell Lines. Proceedings of The American Association of Cancer Research (1996)

Cancer Susceptibility Genes

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Introduction

The emerging discipline of molecular epidemiology seeks to identify biological risk factors that increase an individual's risk of acquiring cancer. More than 70% of all cancers are considered to be due to carcinogen exposures, suggesting that cancer may be highly preventable (Doll and Peto, 1981). Among the most potent of known carcinogens is tobacco smoke, which increases lung cancer risk by more than 14-fold (Doll and Peto, 1978), but the effects of tobacco can vary widely. For example, only about 10% of heavy smokers ever develop lung cancer. It is plausible that these people possess a combination of genetic susceptibility factors that increase their risk following exposure. In an attempt to understand the risks related to specific individuals, a major area of substantial research relates to genetic susceptibilities and biomarker development.

Two fundamental principles that underlie current studies of molecular epidemiology relate to the complexity of the carcinogenesis process and the fact that individuals vary in response to carcinogenic exposures. For the first, cancer is a multistage process where behind each stage are several genetic events, for which there are many steps leading to these events. These events might be triggered by exogenous exposures (e.g., chemicals, radiation or viruses), endogenous exposures (e.g., oxy-radicals) or enzymatic errors (e.g., polymerase or recombinase 9oinfidelity). Along with each of these triggers, several other processes must occur or go awry. For example, for chemical exposures, developing simply from exposure is not sufficient for cancer. Before a cancer causing mutation occurs, a potential carcinogen has to be absorbed, undergo metabolic activation (which frequently requires several enzymatic steps), escape detoxification, be

transported to a target organ, adduct a critical protooncogene or tumor suppressor gene, escape DNA repair, and escape other mechanisms to control the damage such as cell death. Thus, the multistage process of carcinogenesis is a very complicated process consisting of multiple steps and pathways. This complexity leads to difficulties in the design and interpretation of studies, and the need for formulate a priori hypotheses are critical.

The second fundamental principle of molecular epidemiology is based upon data showing interindividual variation (Harris, 1989) in response to carcinogen exposure and carcinogenic processes. In fact, the population is actually quite heterogeneous. Different ages, gender, race, ethnicity, lifestyle (e.g., tobacco use, alcohol, exercise), diet, occupation, and recreational activities all might contribute to differences in responses to environmental agents, although the relative effects of these are currently unknown. Equally important are inherited differences in the host's response to specific exposures, such as in one's metabolic capacity to activate or detoxify carcinogens, and/or repair DNA damage. We are thus further challenged by the paradigm that a combination of exposures and susceptibilities leads to one type of cancer in one person, but another type in a different person, and that different combinations of exposures and susceptibilities can lead to the same type of cancer in different people.

Our approach to studying carcinogenesis in epidemiology is rapidly changing. We are beginning to categorize our genes differently. Specifically, genes have caretaker functions, while others can be thought of as gatekeepers (Kinzler and Vogelstein, 1997). The former category includes genes that maintain basic cellular and genomic integrity, such as DNA repair, carcinogen

metabolism, and DNA replication. The latter category includes genes that regulate cell cycle or govern programmed cell death. Dysfunctional caretaker genes increase the probability of mutations in gatekeeper genes, which are necessary to initiate the molecular pathogenesis of cancer. As we classify genes according to this model, rather than whether genes are protooncogenes or tumor suppressor genes, we will also consider gene-gene interactions for cancer risk from a different perspective.

Genetic variation leads to varying degrees of cancer risk. The frequency for "at risk" genetic variants range from rare to common, as is the potential importance and penetrance (i.e., who is affected). Low penetrant genes typically affect common sporadic cancers, while high penetrance genes cause the rare family cancer syndromes. The relative roles for inherited susceptibility and carcinogen exposure can be very different for familial and common sporadic cancers (Table 1) (Caporaso and Goldstein, 1995). Examples of highly penetrant rare mutations are inheritance of p53 mutations in Li-Fraumeni syndrome families and BRCA1 mutations in breast cancer families. Nonetheless, these rare mutations account for less than 1% of all human cancer, and carcinogen exposure plays a small role in these syndromes (Fearon, 1997). In contrast, polymorphisms for carcinogen metabolizing genes are examples of low penetrance traits with relatively small increased risks for the individual, but have important public health consequences because the risk affects many people. (A polymorphism is a genetic trait that occurs in at least 1% of a population.) Examples include genetic risk factors for either colon or tobacco-related lung cancer. For these commonly-occurring sporadic cancers, carcinogen exposures (exogenous and endogenous) play a critical role, and gene-environment interactions are

common.

TABLE 1

Inherited susceptibilities to cancer are due to variations in the genetic code that alter protein function or localization. A common approach to studying cancer risk is through the determination of genetic polymorphisms. This difference in sequence might be a single nucleic acid base change that changes the protein coding, resulting in a different amino acid sequence and attendant change in function. The possible variations, however, range from single nucleic acid base changes to whole deletions of a gene. Polymorphic variations occur commonly within the genome, although most are either silent because they do not affect amino acid changes or they occur in a noncoding region of the gene. The most interesting polymorphisms to study, however, are the ones that result in changes in protein quantity or function, because those studies can be based on biologically-derived hypotheses.

Genetic susceptibility can be assessed either phenotypically (measuring the resultant enzymatic function) or genotypically (determining the genetic code). Phenotypic assays may include determining enzymatic activity by administering probe drugs to individuals and measuring urinary metabolites, assessing carcinogen metabolic capacity in cultured lymphocytes, or establishing the ratios of endogenously produced substances, such as estrogen metabolite ratios. Using a genetic-based assay to assess cancer risk is generally preferable because DNA is easier to obtain and the assays are technically simpler. However, phenotypes result from the effects of

several genes, and may not be adequately characterized by only one genetic assay. Therefore, there is a role for both genetic- and phenotype-based assays in research studies. Examples of frequently studied genetic polymorphisms in sporadic cancers are the N-acetyltransferase 2 (NAT2), glutathione-S-transferase M1 (GSTM1) and cytochrome P450 1A1 (CYP1A1) genes. The "at-risk" genetic variants for these genes in the United States population (Caucasians and African Americans) are approximately 50%, 50% and <10%, respectively.

Low Risk Penetrance Genes: Genetic Polymorphisms for Carcinogen Activation

Among the most intense areas of polymorphism studies involves genes that govern carcinogen metabolic activation and detoxification. Carcinogens that enter the body from exogenous sources, or produced endogenously, are typically recognized as foreign agents that need to be excreted. For most carcinogens, this process involves a series of steps that make the carcinogen water soluble and/or conjugated to a carrier molecule. The first step is that carcinogens are oxidatively metabolized to reactive electrophillic intermediates by cytochrome P450s (CYP450) or other enzymes (phase I reaction), followed by conjugation reactions (phase II reactions) that detoxify the compound. A polymorphism within a phase I enzyme that results in increased production of an electrophillic intermediate or one in a detoxification enzyme that decreases detoxification can result in elevated levels of reactive intermediates that can damage DNA (Figure 1).

FIGURE 1

Cytochrome p450 (CYP450) enzymes are heme-containing proteins, which can play either an activating or detoxifying role, depending on the CYP450 and the substrate. The CYP450 nomenclature (Nelson et al. 1993) is based on the degree of genetic similarity, chromosomal location and substrate specificity. For human genes, an Arabic numeral and letter follow the "CYP" designation, indicating the family and subfamily of the gene (e.g., CYP-1A). A gene family shares greater than 40% gene homology, has the same number of exons, and similar intron-exon boundaries. Sub-families are located in the same gene cluster and are non-segregating (e.g., CYP1A1, CYP1A2). Mammalian sequences within the same subfamily are always >55% identical. The last Arabic numeral designation usually is based on the order in which the gene sequence of the particular enzyme become known, and on substrate preferences.

Cytochrome P450 1A1: CYP1A1 plays an important role in the activation of potential human carcinogens and also metabolizes estrogens. CYP1A1 substrates include polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene, a carcinogen in tobacco smoke. The CYP1A1 gene is primarily expressed in extra-hepatic tissues, such as the lung, implying its importance in activating carcinogens locally within the target organ. The level of CYP1A1 is regulated by the aromatic hydrocarbon receptor (Ah-receptor), which is stimulated by tobacco smoke, PAHs, organochlorine compounds, steroids and other compounds. Thus, it is presumed that some carcinogens, through the induction of CYP1A1, can lead to greater activation of those same carcinogens, heightening cancer risk. When an inducer binds to the Ah-receptor in the cytoplasm, the compound-receptor complex translocates to the nucleus and up-regulates the expression of

CYP1A1 by binding to the Ah-responsive elements in the 5' flanking region of the CYP1A1 (Catteau et al. 1995; Fujii-Kuriyama et al. 1995). Genetic variants of this receptors that govern CYP1A1 inducibility have not yet been identified (Kawajiri et al. 1995), although the large degree of interindividual variation for inducibility makes it likely that such genetic variants exist.

CYP1A1 enzyme activity varies greatly among people, more than 100-fold, and this variation is thought to lead to cancer risk. Several *CYP1A1* candidate genetic polymorphisms exist, although none have been conclusively established that can alter the function of the enzyme. One commonly studied genetic polymorphism is found in the 3' region of the gene, outside the coding area. It occurs at a restriction enzyme sensitive site, so that it is easily identified with a restriction enzyme digestion, specifically *MSP*1. Associations have been shown between Msp1 genotype and CYP1A1 activity by some investigators (Landi et al. 1994), but not by others (Cosma et al. 1993). This enzyme polymorphism has been most closely associated with cancer risk, but primarily in Japanese. Kawajiri and coworkers (Kawajiri et al. 1990) noted a 3-fold higher risk or lung cancer with one variant. Further, it was found that there was an interaction between this polymorphism and smoking for lung cancer risk (Nakachi et al. 1991; Okada et al. 1995), where the combination of the homozygous minor allele and smoking yielded odds ratios similar to having one of the other genotypes and a greater smoking history. The polymorphism increased risk for both squamous cell and adenocarcinomas (Hayashi et al. 1991; Nakachi et al. 1995). One report associates this polymorphism with increased stages of disease (Okada et al. 1995), and recent data suggest that CYP1A1 genetic polymorphisms predict the presence of p53 mutations (Kawajiri et al. 1996). Also in this study group there was a 4.5-fold increase in risk

(95% CI= 1.64, 12.26) for smokers who carried a rare allele for CYP1A1 genetic polymorphisms (Kawajiri et al. 1996). Overall, similar findings have not been found in Western populations (Shields et al. 1993; Alexandrie et al. 1994; Hirvonen et al. 1992; Tefre et al. 1991). One explanation for this discrepancy is that the frequency of the "at-risk allele is three times greater in Japanese compared with Westerners, so that this gene might still play a role in Westerners, but our epidemiology studies might be too insensitive. Another possible reason for the differences in findings between different races may be that the Msp1 polymorphism is a marker for another genetic variation, which only occurs in Japanese (Wedlund et al. 1994).

A polymorphism exists in exon 7 of *CYP1A1*, which codes for a valine substitution for isoleucine. This site is located in the heme-binding region of the enzyme. Catalytic activity of the variants has been studied, and although the substitution is in a critical area of the gene, there is no difference in catalytic efficiency (Zhang et al. 1996; Persson, Johansson and Ingelman-Sundberg, 1997). This polymorphism has been associated with lung cancer in Japanese (Kawajiri et al. 1993), and in Brazilians (Sugimura et al. 1995), but not in Caucasians (Cascorbi, Brockmoller and Roots, 1996; Alexandrie et al. 1994; Hirvonen et al. 1992). Associations between CYP1A1 genotype and breast cancer have been reported in lighter smokers (Ambrosone et al. 1995; Ishibe et al. 1997), but these findings are considered preliminary and other studies have been negative (Rebbeck, Godwin and Buetow, 1996).

The third *CYP1A1* polymorphism exists only in African-Americans (Crofts et al. 1993). It was thought to be associated with adenocarcinoma of the lung (Eliopoulos et al. 1995), but

other studies could not confirm this finding (Kelsey, Wiencke and Spitz, 1994; Taioli et al. 1995a; London et al. 1995b). Taoli and Garte suggest (Taioli and Garte, 1996)) suggest that possible differences in detecting the association of the genotype with histologic type may be due to appropriate selection of controls, size of the studies, or effect of adjustment for smoking, but it may also be a spurious finding. This genetic variant also might be a risk factor for breast cancer in African Americans (Taioli et al. 1995b).

Glutathione -S-Transferases: These enzymes play an important role in detoxification. They conjugate reactive carcinogen and carcinogen metabolites with glutathione, thereby increasing water solubility and enhancing excretion. Five genes for glutathione-S-transferases have been identified: alpha, kappa, mu, pi, and theta. Of these, genetic polymorphisms in *GSTM1* have been extensively studied, because in approximately one half the population the gene is deleted. In those people, there is an inability to detoxify specific carcinogens. These persons have decreased DNA damage in lung (Shields et al. 1993; Kato et al. 1995; Rothman et al. 1995) and placenta (Topinka et al. 1997) (implicating this gene in teratogenesis or cancer risk). The *GSTM1* null genotype is associated with lung cancer in several studies (Seidegard et al. 1990; Zhong et al. 1991; Nakajima et al. 1995; London et al. 1995a), and a meta-analysis was supportive of increased cancer risk (Rebbeck, 1997). This genotype also predicts the presence of p53 mutations within those cancers (Ryberg et al. 1994). Separately, the occurrence of the *GSTM1* null genotype is related to bladder cancer risk (Anwar et al. 1996; Brockmoller et al. 1996), and p53 mutations within those tumors (Brockmöller et al. 1993). GSTM1 also modulates lung GSTM3 levels (Anttila et al. 1995; Nakajima et al. 1995) and CYP1A1 transcription (Vaury et al. 1995), so that the GSTM1-

related cancer risk may be due to detoxification and induction of other genes.

There has been increasing attention to polymorphisms of GST-P1, where there is a single amino acid (isoleucine /AA or valine/GG) at position 104 in some persons. This change alters the conformation of the catalytic site, producing differences in reactivity toward substrates (Hu et al. 1997). This variation has been associated with increased DNA damage in human lung tissues, male lung cancer risk (Ryberg et al. 1997) and oral cavity cancer (Matthias et al. 1998).

GSTT1 is another polymorphism where the entire gene is deleted in some persons. This deletion is associated with increased DNA damage in cultured lymphocytes exposed to 1,3-butadiene metabolites in vitro (Norppa et al. 1995) and in vivo (Christensen et al. 1998), which is a potent lung carcinogen in laboratory animals and present in tobacco smoke. Evidence for an association of GSTT1 and lung cancer is lacking, however, but there is some evidence for esophageal, laryngeal cancers and skin cancers (Hung et al. 1997; Hung et al. 1997; Lear et al. 1997).

Arylamine Transferases: These enzymes are involved in the metabolism of aromatic amines. Aryl aromatic amines, such as 4-aminobiphenyl, are present in cigarette smoke and the workplace, and have been implicated in bladder and breast cancers. Heterocyclic aromatic amines, formed from the overcooking of foods such as meat, chicken and fish, are thought to be involved in colon carcinogenesis. Two arylamine transferases have been implicated in human cancer risk. NAT1 plays a bioactivation role as a phase I enzyme, while NAT2 both can bioactivate and detoxify,

depending on the substrate.

The NAT2 genetic polymorphism was among the first to be described because it governs the acetylation of isoniazid for tuberculosis treatment, where slow acetylators have increased chances of hepatic and neurological toxicity. The percentage of NAT2 slow acetylators among different ethnic or racial groups range from 20 to 80 percent. The slow acetylator phenotype has been linked to occupationally-induced bladder cancer in dye workers exposed to large amounts of N-substituted aryl compounds (Cartwright et al. 1982). This is believed to be related to liver activation by the cytochrome P4501A2 gene, subsequent transport of reactive metabolites to the bladder via urine and then decreased detoxification through NAT2 activity. To support this hypothesis in humans, one study (Landi et al. 1996) has shown that rapid oxidizers of CYP1A2 combined with NAT2 slow-acetylation phenotypes showed the highest level of 4-aminobiphenyl-hemoglobin adducts. An increased risk for rapid acetylators has been reported in studies of colon cancer (Lang et al. 1986), where there is a hypothesized relationship to activation of heterocyclic amines by the NAT2 gene in colonic epithelium. Then, both CYP1A2 and NAT2 bioactivate these compounds leading to more DNA damage. For example, persons with higher activity by CYP1A2 and rapid acetylation by NAT2 had the highest risks for colorectal neoplasia in people who consumed well-done red meat (Lang et al. 1994). Separately, tobacco smoking was associated with breast cancer in Caucasian postmenopausal women who were NAT2 slow acetylators, and predictably had a decreased capacity to detoxify aromatic amines (Ambrosone et al. 1996). Although a prospective study of nurses (Hunter et al. 1997) seemingly provided conflicting data, there were differences in data analysis and study size. Other

studies are nearing completion so that more evidence soon will be available.

NAT2 and cigarette smoking is a good example of a gene-environment interaction. It was originally shown that the level of 4-aminobiphenyl hemoglobin adducts, where 4-aminobiphenyl is a constituent of cigarette smoke, was correlated with levels of cigarette smoking (Vineis et al. 1990). In fact, it was found that the levels correlated with the type of cigarette smoked (black versus blond), but that in all cases, levels were higher in persons who were phenotypically slow acetylators. It was hypothesized that the decreased detoxification by the NAT2 in the liver led to an increased number of reactive metabolites that were available to adduct hemoglobin. The data have undergone further analysis (Vineis et al. 1994), where levels of cigarette smoking were considered in study subjects by measuring urinary nicotine and cotinine. The analysis suggested that persons with passive tobacco smoke exposure and who were slow acetylators had a proportional greater risk of forming adducts compared with active tobacco smokers.

The *NAT1* gene polymorphism is located in a polyadenylation site, which was suggested to be associated with higher tissue levels of the enzyme (Badawi et al. 1996), presumably by elevating NAT1 mRNA levels. NAT1 activity in bladder and carcinogen-DNA adduct levels in individuals heterozygous for the *10 allele was 2-fold higher than what was observed in individuals homozygous for the *4 allele. The highest adduct levels were observed in individuals with NAT1 rapid genotypes (*10 allele) combined with NAT2 slow genotypes. This variant is associated with increased risk for colorectal cancer (Bell et al. 1995).

High Penetrance Genes: Familial Cancer Syndromes

Familial Cancer accounts for about 1% of all cancers. These are caused by inheritance of mutated cancer susceptibility genes that are highly penetrant, where the chances of developing cancer in a person with the mutation can be as high as 90%. Although these genes cause only a fraction of total cancers, and so may not have a clear public health importance, they are clearly devastating to those cancer families and research on familial cancers has significantly advanced both basic science and clinical aspects of oncology. In many cases, the same genes are sporadically mutated in common cancers. These genes are typically involved in cell-cycle control, DNA repair, transcription regulation, cell-cell or cell-matrix adhesion, cytoskeletal architecture, and signal transduction.

There are different ways to conceptualize the roles of genes in cancer susceptibility. One way is to consider genes as proto-oncogenes and tumor suppressor genes. Mutations in proto-oncogenes are generally inherited in an autosomal dominant fashion, and therefore a mutation in a single allele, whether acquired or inherited may be adequate to initiate disease. The mutations in oncogenes produce a gain of function, or increase the function of the gene's protein product, which thereby increases the risk of cancer. Mutations in tumor suppressor genes generally act recessively. Mutations in both alleles of a gene need to occur. In the case of a tumor suppressor gene, the mutations result in a loss of function or an abrogation of the activity of the gene's protein product. The second way to conceptualize and categorize the role of genes in cancer was recently proposed (Kinzler and Vogelstein, 1997), whereby we consider them as

caretaker and gatekeeper genes. Here, we acknowledge their respective roles as a caretaker in maintaining genomic integrity and as gatekeepers affecting cellular proliferation. Some examples of caretaker genes are those that are involved in either DNA repair, while examples of gatekeeper genes are those involved in cell cycle control and DNA replication. Dysfunctional caretaker genes increase the probability of mutations in gatekeeper genes, which are necessary to initiate the molecular pathogenesis of cancer. It is interesting that the carcinogenic effects of this dysfunction appear to be tissue specific and lead to cancer only in those organs, even though these genes are expressed in many different organs.

Retinoblastoma: Initial insights into the mechanisms of tumor suppressor genes were gained from early studies of retinoblastoma. Patients without family histories of retinoblastoma usually develop it only in a single eye, whereas in persons with a family history, the cancers occur in both eyes. Also, inherited disease usually occurs much earlier in life than sporadic cancer. Mapping studies for the retinoblastoma susceptibility gene (Rb) revealed that there was a loss of functional gene in both familial and sporadic disease. In most cases, the wild-type allele was lost in the tumor. This observation led Knudson (Cooper, 1995) to propose a "two-hit" hypothesis where a mutation in a tumor suppressor gene either occurs through inheritance or induced through carcinogen exposure. A second mutation resulting in the loss of the wild-type allele then occurs which obliterates the function of the gene.

The p53 tumor suppressor gene: p53 is a 53-kDa nuclear phosphoprotein tumor suppressor gene that is mutated in more than 50% of sporadic cancer cases. Inherited p53 germline

mutations only account for a few cases of familial cancer, and occurs in 60% of Li-Fraumeni Syndrome families and about 25% of "Li-Fraumeni-like" syndrome families. The gene is located at chromosome 17p. p53 functions in cell cycle control, apoptosis, DNA repair and transcriptional activation. Li Fraumeni syndrome is characterized by multiple primary tumors occurring relatively early in life, including early onset breast cancer, childhood soft-tissue sarcomas, leukemias, adrenocortical carcinomas and central nervous system tumors (Evans and Lozano, 1997). The classical definition of the tumor requires that a proband be diagnosed with a sarcoma before age 45, a first degree relative with cancer before age 45, and a first or second degree relative with either a sarcoma at any age or any cancer before 45. The distributions of germline mutations in p53 appear to be similar to the distribution in sporadic cancers (Kleihues et al. 1997). The majority of germline mutations occur within exons 5, 7 and 8 (Varley et al. 1996; Saeki et al. 1997).

Mismatch Repair Genes hMSH2 and hMLH1: These genes cause hereditary non-polyposis colorectal cancer (HNPCC), also known as the Lynch syndromes, which is characterized by the absence of polyposis, early age of diagnosis, high prevalence of right sided colon tumors and susceptibility to other primary malignancies (e.g., uterine, ovarian, stomach, pancreas, and breast). Mutations in hMSH2 and hMLH1 DNA repair genes have been found to account for 65% of the cases of HNPCC (Kinzler and Vogelstein, 1996; Edelmann et al. 1997). Splicing defects producing alternate splicing of exons 9,10, 15, 16, 17 in hMLH1 and exon 5 in hMSH2 account for a substantial fraction of the germline mutations in hMSH2 and hMLH1 in HNPCC (Wijnen et al. 1996). Two other mismatch repair genes, hPMS1 and hPMS2 (human post-meiotic

segregation 1 and 2) located on chromosomes 2q31-32 and 7p22 may be involved in the remainder of the cases (Nicolaides et al. 1995). Germline mutations in TGF-β II receptor genes also may also play a role in HNPCC (Lu et al. 1998).

There is an interesting gene-dose effect for alterations in MSH2 and MLH1. Replication errors are the hallmark of these tumors, where the effect of defective DNA repair is manifested in the number of repeating nucleotide sequences. Intra-tumor heterogeneity occurs, where there are mild or severe forms, depending on the number of repeats (Habano, Sugai and Nakamura, 1998). The loss of a single MSH2 and MLH allele may have only a mild effect on DNA repair capability and result in a mild replication error (RER+) phenotype. But, in tumor cells where both alleles are affected, or one deficient allele occurs with a germline mutation, a severe RER+ phenotype is observed, consistent with the two hit hypothesis.

Several specific genetic defects have been reported, but alterations in these genes via genetic polymorphisms might affect sporadic cancer risk too. Such candidate polymorphisms include an intronic germline T>C transition at exon 13 -6 splice acceptor site of hMSH2, found in 25% of healthy Caucasians and 33% of Japanese. This particular variant may increase the sporadic colorectal cancer risk to 3.2-fold (Goessl et al. 1997).

APC gene: Familial adenomatous polyposis (FAP) syndrome is an autosomal dominantly inherited disease that leads to colon cancer. FAP patients have thousands of adenomatous polyps in the colon by the age of 20, and 90% of these patients develop colon cancer by the fifth decade of life

if no treatment is received (Eng and Ponder, 1993). The FAP syndrome also is associated with extra-intestinal manifestations such as congenital hypertrophy of the retinal pigment epithelium (CHRPE), dental lesions as well as a high incidence of desmoid tumors in the colon.

Inherited mutations in the adenomatous polyposis coli (APC) gene is considered an early step in FAP, but somatic mutations in sporadic polyps and cancers also are common. APC protein binds to beta-catenins, which have been shown to interact with proteins involved in cell-cell adhesion (cadherins). Specific domains in APC are involved in beta-catenin binding, and the location of mutations is associated with the phenotypic features of disease (Fodde and Khan, 1995). Approximately 95% of mutations occur in the 5' half of the gene, most of which result in protein truncation (Nagase et al. 1992a; van der Luijt et al. 1996). Patients who have frameshift mutations between codons 1250 and 1330 develop more than 5000 polyps, whereas mutations outside this region generally leads to less than 2000 polyps and milder disease (Nagase et al. 1992b; Friedl et al. 1996). Families with mutations located toward the 5' end of codon 158 are more likely to have heterogeneous features including delayed development of colonic polyposis and colorectal cancer than families with more distal 5'mutations in the gene (Giardiello et al. 1997). Other mutations such as a frameshift mutation and a termination at codons 1862 and 1987 have been associated with greater variability in the number of colorectal adenomas, and mutations at codon 1309 results in thousands of adenomas at a young age and earlier death (Caspari et al. 1995; Gayther et al. 1994).

BRCA1 and BRCA2 tumor suppressor genes: BRCA1 is located on chromosome 17q21.

Although its exact function is unknown, it is likely a transcription factor, suggested by the predicted zinc-finger domain. BRCA2 appears to be involved in DNA repair (Patel et al. 1998)). The BRCA1 gene contains 22 exons encoding a protein 1,863 amino acids. BRCA2 is located on chromosome 13q12-13. It contains 26 exons and encodes a protein 3,418 amino acids in length. Germline mutations in BRCA1 account for about 50% of families with a dominant predisposition to breast cancer and up to 90% of families with breast and ovarian cancer (Szabo and King, 1995; Easton, Ford and Bishop, 1995). Most germline mutations are frameshift or non-sense mutation that result in truncated or inactive protein. In these families, women inherit one mutated gene, and the other is lost during life, supporting its role as a tumor suppressor (Silva et al. 1998; Shen et al. 1998). An interesting finding is that there is a BRCA1 genetic polymorphism (185delAG) and a BRCA2 genetic polymorphism, which are found in approximately 1% of Ashkenazi Jews and leads to a lifetime breast cancer risk of about 45% (Struewing et al. 1997). In African American compared with Caucasian women, different BRCA1 mutations are found (Shen et al. 1998).

RET Gene: The RET gene codes for a tyrosine kinase receptor whose ligand has been identified as glial-cell derived neurotropic factor (Marsh et al. 1997)). Germline mutations in *RET* are found in the syndrome of Multiple Endocrine Neoplasia 2 (MEN2) (Marsh et al. 1996; Frank-Raue et al. 1996; Borrello et al. 1995; Uchino et al. 1998). MEN2 is an autosomal dominant inherited cancer syndrome that is subdivided based on which organs are affected. MEN2A is associated with medullary thyroid carcinoma (MTC) of the parafollicular C cells of thyroid, pheochromocytoma and hyperparathyroidism. MEN2B is similar to form 2A with the

exception the parathyroid disease does not occur. Familial Medullary Thyroid Carcinoma (FMTC) is a form where only medullary thyroid carcinoma is present.

Several types of *RET* mutations have been described, and are associated with different outcomes. Mutations in the extracellular cysteine rich domain of *RET* are found in the majority of families with MEN2A and FMTC (Mulligan et al. 1995; Blank et al. 1996; Eng et al. 1996; Schuffenecker et al. 1998). Persons with codon 618 mutations had a lower frequency of pheochromocytoma and parathyroid cancer, and increased life expectancy (Moers et al. 1996). Mutations at codon 768 (exon 13) and 804 (exon 14) in the intracellular domain of the receptor have been associated with FMTC (Miyauchi et al. 1997; Eng et al. 1996; Fink et al. 1996). Mutations in the tyrosine kinase domain lead to oncogenic activation of RET and the development of FMTC (Pasini et al. 1997). A mutation in codon 918 (exon 16) occurs at the substrate recognition site of the receptor (Eng et al. 1996; Rossel et al. 1995; Maeda et al. 1995; Blank et al. 1996) and has been associated with glaucoma in MEN2B (Mashima et al. 1998).

Mutations in *RET* also have been reported in sporadic cases of MTC (Fink et al. 1996). For example, 29% of tumors contain a somatic mutation at codon 918 and correlates with poor prognosis (Zedenius et al. 1995).

Figure 1. This schema illustrates a common pathway where procarcinogens undergo metabolic processing that generally leads to the production of compounds with increased water solubility and enhanced excretion. Enzyme polymorphisms that increase the oxidative pathway (activation

of the carcinogen), decrease the conjugation (detoxification), or a combination of both can lead to increased levels of the reactive electrophillic intermediates. These intermediates can damage proteins, lipids and DNA. The DNA damage is usually repaired, but if the reactive intermediates overwhelm the repair mechanism, a mutation could result in a gene critical to losing cell cycle control, differentiation or programmed cell death.

Bibliography

Alexandrie, A.-K., Sundberg, M.I., Seidegard, J., Tornling, G. and Rannug, A. 1994, Genetic susceptibility to lung cancer with special emphasis on CYP1A1 and GSTM1: A study on host factors in relation to age at onset, gender and histological cancer types, *Carcinogenesis* **15,** 1785.

Ambrosone, C.B., Freudenheim, J.L., Graham, S., Marshall, J.R., Vena, J.E., Brasure, J.R., Laughlin, R., Nemoto, T., Michalek, A.M., Harrington, A., Ford, T.D. and Shields, P.G. 1995, Cytochrome P4501A1 and glutathione S-transferase (M1) genetic polymorphisms and postmenopausal breast cancer risk, *Cancer Res* **55,** 3483.

Ambrosone, C.B., Freudenheim, J.L., Graham, S., Marshall, J.R., Vena, J.E., Brasure, J.R., Michalek, A.M., Laughlin, R., Nemoto, T., Gillenwater, K.A., Harrington, A.M. and Shields, P.G. 1996, Cigarette smoking, N-acetyltransferase 2 genetic polymorphisms, and breast cancer risk, *JAMA* **276,** 1494.

Anttila, S., Luostarinen, L., Hirvonen, A., Elovaara, E., Karjalainen, A., Nurminen, T., Hayes, J.D., Vainio, H. and Ketterer, B. 1995, Pulmonary expression of glutathione S-transferase M3 in lung cancer patients: association with GSTM1 polymorphism, smoking, and asbestos exposure, *Cancer Res* **55,** 3305.

Anwar, W.A., Abdel-Rahman, S.Z., El-Zein, R.A., Mostafa, H.M. and Au, W.W. 1996, Genetic

polymorphism of GSTM1, CYP2E1 and CYP2D6 in Egyptian bladder cancer patients, *Carcinogenesis* **17,** 1923.

Badawi, A.F., S.J. Stern, N.P. Lang and F.F. Kadlubar, 1996, Cytochrome P-450 and aceyltransferase expression as biomarkers of carcinogen-DNA adduct levels and human cancer susceptibility, in: Genetics and Cancer Susceptibility: Implications for Risk Assessment, eds. C. Walker, J. Groopman, T.J. Slaga and A. Klein-Szanto (Wiley-Liss, Inc. New York) p. 109.

Bell, D.A., Stephens, E.A., Castranio, T., Umbach, D.M., Watson, M., Deakin, M., Elder, J., Hendrickse, C., Duncan, H. and Strange, R.C. 1995, Polyadenylation polymorphism in the acetyltransferase 1 gene (NAT1) increases risk of colorectal cancer, *Cancer Res* **55,** 3537.

Blank, R.D., Sklar, C.A., Dimich, A.B., LaQuaglia, M.P. and Brennan, M.F. 1996, Clinical presentations and RET protooncogene mutations in seven multiple endocrine neoplasia type 2 kindreds, *Cancer* **78,** 1996.

Borrello, M.G., Smith, D.P., Pasini, B., Bongarzone, I., Greco, A., Lorenzo, M.J., Arighi, E., Miranda, C., Eng, C. and Alberti, L. 1995, RET activation by germline MEN2A and MEN2B mutations, *Oncogene* **11,** 2419.

Brockmoller, J., Cascorbi, I., Kerb, R. and Roots, I. 1996, Combined analysis of inherited polymorphisms in arylamine N-acetyltransferase 2, glutathione S-transferases M1 and T1,

microsomal epoxide hydrolase, and cytochrome P450 enzymes as modulators of bladder cancer risk, *Cancer Res* **56,** 3915.

Brockmöller, J., Kerb, R., Drakoulis, N., Nitz, M. and Roots, I. 1993, Genotype and phenotype of glutathione S-transferase class mu isoenzymes mu and psi in lung cancer patients and controls, *Cancer Res* **53,** 1004.

Caporaso, N. and Goldstein, A. 1995, Cancer genes: single and susceptibility: exposing the difference, *Pharmacogen* **5,** 59.

Cartwright, R.A., Glashan, R.W., Rogers, H.J., Ahmad, R.A., Barham-Hall, D., Higgins, E. and Kahn, M.A. 1982, Role of N-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer, *Lancet* **2,** 842.

Cascorbi, I., Brockmoller, J. and Roots, I. 1996, A C4887A polymorphism in exon 7 of human CYP1A1: population frequency, mutation linkages, and impact on lung cancer susceptibility, *Cancer Res* **56,** 4965.

Caspari, R., Olschwang, S., Friedl, W., Mandl, M., Boisson, C., Boker, T., Augustin, A., Kadmon, M., Moslein, G. and Thomas, G. 1995, Familial adenomatous polyposis: desmoid tumours and lack of ophthalmic lesions (CHRPE) associated with APC mutations beyond codon 1444, *Hum Mol. Genet* **4,** 337.

Catteau, A., Douriez, E., Beaune, P., Poisson, N., Bonaiti-Pellie, C. and Laurent, P. 1995, Genetic polymorphism of induction of CYP1A1 (EROD) activity, *Pharmacogenetics.* **5,** 110.

Christensen, E.R., Cunningham, J.M., Tester, D.J., Roche, P.C., Burgart, L.J. and Thibodeau, S.N. 1998, Hypermethylation of the hMLH1 promoter in colon cancer with microsatelite instability, *Proc. Am. Assoc. Cancer Res.* **39,** 460.

Cooper, G.M. 1995, Retinoblastoma and the discovery of tumor suppressor genes, in: Oncogenes, Vol.2nd, ed. J.E. Burns (Jones and Bartlett Publ. Boston) p. 126.

Cosma, G., Crofts, F., Currie, D., Wirgin, I., Toniolo, P. and Garte, S.J. 1993, Racial differences in restriction fragment length polymorphisms and messenger RNA inducibility of the human CYP1A1 gene, *Cancer Epidemiol Biomarkers Prev.* **2,** 53.

Crofts, F., Cosma, G.N., Currie, D., Taioli, E., Toniolo, P. and Garte, S.J. 1993, A novel CYP1A1 gene polymorphism in African-Americans, *Carcinogenesis* **14,** 1729.

Doll, R. and Peto, R. 1978, Cigarette smoking and bronchial carcinoma: dose and time relationships among regular smokers and lifelong non-smokers, *J. Epidemiol. Community. Health* **32,** 303.

Doll, R. and Peto, R. 1981, The causes of cancer: quantitative estimates of avoidable risks of

cancer in the United States today, *J Natl. Cancer Inst.* **66,** 1191.

Easton, D.F., Ford, D. and Bishop, D.T. 1995, Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium, *Am. J Hum Genet* **56,** 265.

Edelmann, W., Yang, K., Umar, A., Heyer, J., Lau, K., Fan, K., Liedtke, W., Cohen, P.E., Kane, M.F., Lipford, J.R., Yu, N., Crouse, G.F., Pollard, J.W., Kunkel, T., Lipkin, M., Kolodner, R. and Kucherlapati, R. 1997, Mutation in the mismatch repair gene Msh6 causes cancer susceptibility, *Cell* **91,** 467.

Eliopoulos, A.G., Kerr, D.J., Herod, J., Hodgkins, L., Krajewski, S., Reed, J.C. and Young, L.S. 1995, The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2, *Oncogene* **11,** 1217.

Eng, C., Clayton, D., Schuffenecker, I., Lenoir, G., Cote, G., Gagel, R.F., van Amstel, H.K., Lips, C.J., Nishisho, I., Takai, S.I., Marsh, D.J., Robinson, B.G., Frank-Raue, K., Raue, F., Xue, F., Noll, W.W., Romei, C., Pacini, F., Fink, M., Niederle, B., Zedenius, J., Nordenskjold, M., Komminoth, P., Hendy, G.N. and Mulligan, L.M. 1996, The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis, *JAMA* **276,** 1575.

Eng, C. and Ponder, B.A. 1993, The role of gene mutations in the genesis of familial cancers,

FASEB J **7,** 910.

Evans, S.C. and Lozano, G. 1997, The Li-Fraumeni syndrome: an inherited susceptibility to cancer, *Mol. Med Today.* **3,** 390.

Fearon, E.R. 1997, Human cancer syndromes: clues to the origin and nature of cancer, *Science* **278,** 1043.

Fink, M., Weinhusel, A., Niederle, B. and Haas, O.A. 1996, Distinction between sporadic and hereditary medullary thyroid carcinoma (MTC) by mutation analysis of the RET proto-oncogene. "Study Group Multiple Endocrine Neoplasia Austria (SMENA)", *Int. J Cancer* **69,** 312.

Fodde, R. and Khan, P.M. 1995, Genotype-phenotype correlations at the adenomatous polyposis coli (APC) gene, *Crit. Rev Oncog.* **6,** 291.

Frank-Raue, K., Hoppner, W., Frilling, A., Kotzerke, J., Dralle, H., Haase, R., Mann, K., Seif, F., Kirchner, R., Rendl, J., Deckart, H.F., Ritter, M.M., Hampel, R., Klempa, J., Scholz, G.H. and Raue, F. 1996, Mutations of the ret protooncogene in German multiple endocrine neoplasia families: relation between genotype and phenotype. German Medullary Thyroid Carcinoma Study Group, *J Clin Endocrinol Metab.* **81,** 1780.

Friedl, W., Meuschel, S., Caspari, R., Lamberti, C., Krieger, S., Sengteller, M. and Propping, P.

1996, Attenuated familial adenomatous polyposis due to a mutation in the 3' part of the APC gene. A clue for understanding the function of the APC protein, *Hum Genet* **97,** 579.

Fujii-Kuriyama, Y., Ema, M., Mimura, J., Matsushita, N. and Sogawa, K. 1995, Polymorphic forms of the Ah receptor and induction of the CYP1A1 gene, *Pharmacogenetics.* **5 Spec No,** S149.

Gayther, S.A., Wells, D., SenGupta, S.B., Chapman, P., Neale, K., Tsioupra, K. and Delhanty, J.D. 1994, Regionally clustered APC mutations are associated with a severe phenotype and occur at a high frequency in new mutation cases of adenomatous polyposis coli, *Hum Mol. Genet* **3,** 53.

Giardiello, F.M., Brensinger, J.D., Luce, M.C., Petersen, G.M., Cayouette, M.C., Krush, A.J., Bacon, J.A., Booker, S.V., Bufill, J.A. and Hamilton, S.R. 1997, Phenotypic expression of disease in families that have mutations in the 5' region of the adenomatous polyposis coli gene, *Ann Intern. Med* **126,** 514.

Goessl, C., Plaschke, J., Pistorius, S., Hahn, M., Frank, S., Hampl, M., Gorgens, H., Koch, R., Saeger, H.D. and Schackert, H.K. 1997, An intronic germline transition in the HNPCC gene hMSH2 is associated with sporadic colorectal cancer, *Eur J Cancer* **33,** 1869.

Habano, W., Sugai, T. and Nakamura, S. 1998, Mismatch repair deficiency leads to a unique mode of colorectal tumorigenesis characterized by intratumoral heterogeneity, *Oncogene* **16,**

1259.

Harris, C.C. 1989, Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair, *Carcinogenesis* **10,** 1563.

Hayashi, S., Watanabe, J., Nakachi, K. and Kawajiri, K. 1991, Genetic linkage of lung cancer-associated MspI polymorphisms with amino acid replacement in the heme binding region of the human cytochrome P450IA1 gene, *J Biochem. (Tokyo).* **110,** 407.

Hirvonen, A., Husgafvel-Pursiainen, K., Karjalainen, A., Anttila, S. and Vainio, H. 1992, Point-mutational Msp1 and Ile-Val polymorphisms closely linked in the CYP1A1 gene: lack of association with susceptibility to lung cancer in a Finnish study population, *CEBP* **1,** 485.

Hu, X., O'Donnell, R., Srivastava, S.K., Xia, H., Zimniak, P., Nanduri, B., Bleicher, R.J., Awasthi, S., Awasthi, Y.C., Ji, X. and Singh, S.V. 1997, Active site architecture of polymorphic forms of human glutathione S-transferase P1-1 accounts for their enantioselectivity and disparate activity in the glutathione conjugation of 7beta,8alpha-dihydroxy-9alpha,10alpha-ox y-7,8,9, 10-tetrahydrobenzo(a)pyrene, *Biochem. Biophys. Res Commun.* **235,** 424.

Hung, H.C., Chuang, J., Chien, Y.C., Chern, H.D., Chiang, C.P., Kuo, Y.S., Hildesheim, A. and Chen, C.J. 1997, Genetic polymorphisms of CYP2E1, GSTM1, and GSTT1; environmental factors and risk of oral cancer, *Cancer Epidemiol Biomarkers Prev.* **6,** 901.

Hunter, D.J., Hankinson, S.E., Hough, H., Gertig, D.M., Garcia-Closas, M., Spiegelman, D., Manson, J.E., Colditz, G.A., Willett, W.C., Speizer, F.E. and Kelsey, K. 1997, A prospective study of NAT2 acetylation genotype, cigarette smoking, and risk of breast cancer, *Carcinogenesis* **18,** 2127.

Ishibe, N., Wiencke, J.K., Zuo, Z.F., McMillan, A., Spitz, M. and Kelsey, K.T. 1997, Susceptibility to lung cancer in light smokers associated with CYP1A1 polymorphisms in Mexican- and African-Americans, *Cancer Epidemiol Biomarkers Prev.* **6,** 1075.

Kato, S., Bowman, E.D., Harrington, A.M., Blomeke, B. and Shields, P.G. 1995, Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms in vivo, *J. Natl. Cancer Inst.* **87,** 902.

Kawajiri, K., Nakachi, K., Imai, K., Yoshii, A., Shinoda, N. and Watanabe, J. 1990, Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P450IA1 gene, *FEBS* **263,** 131.

Kawajiri, K., Nakachi, K., Imai, K., Watanabe, J. and Hayashi, S. 1993, Germ line polymorphisms of p53 and CYP1A1 genes involved in human lung cancer, *Carcinogenesis* **14,** 1085.

Kawajiri, K., Watanabe, J., Eguchi, H., Nakachi, K., Kiyohara, C. and Hayashi, S. 1995, Polymorphisms of human Ah receptor gene are not involved in lung cancer, *Pharmacogenetics.* **5,**

151.

Kawajiri, K., Eguchi, H., Nakachi, K., Sekiya, T. and Yamamoto, M. 1996, Association of CYP1A1 germ line polymorphisms with mutations of the p53 gene in lung cancer, *Cancer Res* **56,** 72.

Kelsey, K.T., Wiencke, J.K. and Spitz, M.R. 1994, A race-specific genetic polymorphism in the CYP1A1 gene is not associated with lung cancer in African Americans, *Carcinogenesis* **15,** 1121.

Kinzler, K.W. and Vogelstein, B. 1996, Lessons from hereditary colorectal cancer, *Cell* **87,** 159.

Kinzler, K.W. and Vogelstein, B. 1997, Gatekeepers and caretakers, *Nature* **386,** 761.

Kleihues, P., Schauble, B., zur Hausen, A., Esteve, J. and Ohgaki, H. 1997, Tumors associated with p53 germline mutations: a synopsis of 91 families, *Am. J Pathol.* **150,** 1.

Landi, M.T., Bertazzi, P.A., Shields, P.G., Clark, G., Lucier, G.W., Garte, S.J., Cosma, G. and Caporaso, N.E. 1994, Association between CYP1A1 genotype, mRNA expression and enzymatic activity in humans, *Pharmacogenetics.* **4,** 242.

Landi, M.T., Zocchetti, C., Bernucci, I., Kadlubar, F.F., Tannenbaum, S., Skipper, P., Bartsch, H., Malaveille, C., Shields, P., Caporaso, N.E. and Vineis, P. 1996, Cytochrome P4501A2:

enzyme induction and genetic control in determining 4-aminobiphenyl-hemoglobin adduct levels, *Cancer Epidemiol Biomarkers Prev.* **5,** 693.

Lang, N.P., Chu, D.Z., Hunter, C.F., Kendall, D.C., Flammang, T.J. and Kadlubar, F.F. 1986, Role of aromatic amine acetyltransferase in human colorectal cancer, *Arch. Surg.* **121,** 1259.

Lang, N.P., Butler, M.A., Massengill, J., Lawson, M., Stotts, R.C., Hauer-Jensen, M. and Kadlubar, F.F. 1994, Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps, *Cancer Epidemiol Biomarkers Prev.* **3,** 675.

Lear, J.T., Smith, A.G., Bowers, B., Heagearty, A.H., Jones, P.W., Gilford, J., Alldersea, J., Strange, R.C. and Fryer, A.A. 1997, Truncal tumor site is associated with high risk of multiple basal cell carcinoma and is influenced by glutathione S-transferase, GSTT1, and cytochrome P450, CYP1A1 genotypes, and their interaction, *J Invest. Dermatol.* **108,** 519.

London, S.J., Daly, A.K., Cooper, J., Navidi, W.C., Carpenter, C.L. and Idle, J.R. 1995a, Polymorphism of glutathione S-transferase M1 and lung cancer risk among African-Americans and Caucasians in Los Angeles County, California, *JNCI* **87,** 1246.

London, S.J., Daly, A.K., Fairbrother, K.S., Holmes, C., Carpenter, C.L., Navidi, W.C. and Idle, J.R. 1995b, Lung cancer risk in African-Americans in relation to a race-specific CYP1A1

polymorphism, *Cancer Res* **55,** 6035.

Lu, S.L., Kawabata, M., Imamura, T., Akiyama, Y., Nomizu, T., Miyazono, K. and Yuasa, Y. 1998, HNPCC associated with germline mutation in the TGF-beta type II receptor gene [letter], *Nat. Genet* **19,** 17.

Maeda, S., Namba, H., Takamura, N., Tanigawa, K., Takahashi, M., Noguchi, S., Nagataki, S., Kanematsu, T. and Yamashita, S. 1995, A single missense mutation in codon 918 of the RET proto-oncogene in sporadic medullary thyroid carcinomas, *Endocr. J* **42,** 245.

Marsh, D.J., Andrew, S.D., Eng, C., Learoyd, D.L., Capes, A.G., Pojer, R., Richardson, A.L., Houghton, C., Mulligan, L.M., Ponder, B.A. and Robinson, B.G. 1996, Germline and somatic mutations in an oncogene: RET mutations in inherited medullary thyroid carcinoma, *Cancer Res* **56,** 1241.

Marsh, D.J., Zheng, Z., Arnold, A., Andrew, S.D., Learoyd, D., Frilling, A., Komminoth, P., Neumann, H.P., Ponder, B.A., Rollins, B.J., Shapiro, G.I., Robinson, B.G., Mulligan, L.M. and Eng, C. 1997, Mutation analysis of glial cell line-derived neurotrophic factor, a ligand for an RET/coreceptor complex, in multiple endocrine neoplasia type 2 and sporadic neuroendocrine tumors, *J Clin Endocrinol Metab.* **82,** 3025.

Mashima, Y., Konishi, M., Yamada, M., Imamura, Y., Nii, S. and Nakamura, Y. 1998, Multiple

endocrine neoplasia 2B with glaucoma associated with codon 918 mutation of the RET proto-oncogene, *Acta Ophthalmol. Scand.* **76,** 114.

Matthias, C., Bockmuhl, U., Jahnke, V., Harries, L.W., Wolf, C.R., Jones, P.W., Alldersea, J., Worrall, S.F., Hand, P., Fryer, A.A. and Strange, R.C. 1998, The glutathione S-transferase GSTP1 polymorphism: effects on susceptibility to oral/pharyngeal and laryngeal carcinomas, *Pharmacogenetics.* **8,** 1.

Miyauchi, A., Egawa, S., Futami, H., Kuma, K., Obara, T. and Yamaguchi, K. 1997, A novel somatic mutation in the RET proto-oncogene in familial medullary thyroid carcinoma with a germline codon 768 mutation, *Jpn. J Cancer Res* **88,** 527.

Moers, A.M., Landsvater, R.M., Schaap, C., Jansen-Schillhorn van Veen, J.M., de Valk, I.A., Blijham, G.H., Hoppener, J.W., Vroom, T.M., van Amstel, H.K. and Lips, C.J. 1996, Familial medullary thyroid carcinoma: not a distinct entity? Genotype-phenotype correlation in a large family, *Am. J Med* **101,** 635.

Mulligan, L.M., Marsh, D.J., Robinson, B.G., Schuffenecker, I., Zedenius, J., Lips, C.J., Gagel, R.F., Takai, S.I., Noll, W.W. and Fink, M. 1995, Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the International RET Mutation Consortium, *J Intern. Med* **238,** 343.

Nagase, H., Miyoshi, Y., Horii, A., Aoki, T., Ogawa, M., Utsunomiya, J., Baba, S., Sasazuki, T. and Nakamura, Y. 1992a, Correlation between the location of germ-line mutations in the APC gene and the number of colorectal polyps in familial adenomatous polyposis patients, *Cancer Res* **52,** 4055.

Nagase, H., Miyoshi, Y., Horii, A., Aoki, T., Petersen, G.M., Vogelstein, B., Maher, E., Ogawa, M., Maruyama, M. and Utsunomiya, J. 1992b, Screening for germ-line mutations in familial adenomatous polyposis patients: 61 new patients and a summary of 150 unrelated patients, *Hum Mutat.* **1,** 467.

Nakachi, K., Imai, K., Hayashi, S., Watanabe, J. and Kawajiri, K. 1991, Genetic susceptibility to squamous cell carcinoma of the lung in relation to cigarette smoking dose, *Cancer Res* **51,** 5177.

Nakachi, K., Hayashi, S., Kawajiri, K. and Imai, K. 1995, Association of cigarette smoking and CYP1A1 polymorphisms with adenocarcinoma of the lung by grades of differentiation, *Carcinogenesis* **16,** 2209.

Nakajima, T., Elovaara, E., Anttila, S., Hirvonen, A., Camus, A.M., Hayes, J.D., Ketterer, B. and Vainio, H. 1995, Expression and polymorphism of glutathione S-transferase in human lungs: risk factors in smoking-related lung cancer, *Carcinogenesis* **16,** 707.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R.,

Gonzalez, F.J., Coon, M.J., Gunsalus, I.C. and Gotoh, O. 1993, The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature, *DNA Cell Biol* **12,** 1.

Nicolaides, N.C., Carter, K.C., Shell, B.K., Papadopoulos, N., Vogelstein, B. and Kinzler, K.W. 1995, Genomic organization of the human PMS2 gene family, *Genomics* **30,** 195.

Norppa, H., Hirvonen, A., Jarventaus, H., Uuskula, M., Tasa, G., Ojajarvi, A. and Sorsa, M. 1995, Role of GSTT1 and GSTM1 genotypes in determining individual sensitivity to sister chromatid exchange induction by diepoxybutane in cultured human lymphocytes, *Carcinogenesis* **16,** 1261.

Okada, T., Kawashima, K., Fukushi, S., Minakuchi, T. and Nishimura, S. 1995, Association between a cytochrome P450 CYPIA1 genotype and incidence of lung cancer, *Pharmacogen* **4,** 333.

Pasini, A., Geneste, O., Legrand, P., Schlumberger, M., Rossel, M., Fournier, L., Rudkin, B.B., Schuffenecker, I., Lenoir, G.M. and Billaud, M. 1997, Oncogenic activation of RET by two distinct FMTC mutations affecting the tyrosine kinase domain, *Oncogene* **15,** 393.

Patel, K.J., Yu, V.P.C.C., Lee, H., Corcoran, A., Thistlethwaite, F.C., Evans, M.J., Colledge, W.H., Friedman, L.S., Ponder, B.A. and Venkitaraman, A.R. 1998, Involvement of Brca2 in

DNA repair, *Molec. Cell* **1,** 347.

Persson, I., Johansson, I. and Ingelman-Sundberg, M. 1997, In vitro kinetics of two human CYP1A1 variant enzymes suggested to be associated with interindividual differences in cancer susceptibility, *Biochem. Biophys. Res Commun.* **231,** 227.

Rebbeck, T.R. 1997, Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility, *Cancer Epidemiol Biomarkers Prev.* **6,** 733.

Rebbeck, T.R., Godwin, A.K. and Buetow, K.H. 1996, Variability in loss of constitutional heterozygosity across loci and among individuals: association with candidate genes in ductal breast carcinoma, *Mol. Carcinog.* **17,** 117.

Rossel, M., Schuffenecker, I., Schlumberger, M., Bonnardel, C., Modigliani, E., Gardet, P., Navarro, J., Luo, Y., Romeo, G. and Lenoir, G. 1995, Detection of a germline mutation at codon 918 of the RET proto-oncogene in French MEN 2B families, *Hum Genet* **95,** 403.

Rothman, N., Shields, P.G., Poirier, M.C., Harrington, A.M., Ford, D.P. and Strickland, P.T. 1995, The impact of glutathione s-transferase M1 and cytochrome P450 1A1 genotypes on white-blood-cell polycyclic aromatic hydrocarbon-DNA adduct levels in humans, *Mol. Carcinog.* **14,** 63.

Ryberg, D., Kure, E., Lystad, S., Skaug, V., Stangeland, L., Mercy, I., Borresen, A.L. and Haugen, A. 1994, p53-mutations in lung tumors. Relationship to putative susceptibility markers for cancer, *Cancer Res.* **54,** 1551.

Ryberg, D., Skaug, V., Hewer, A., Phillips, D.H., Harries, L.W., Wolf, C.R., Ogreid, D., Ulvik, A., Vu, P. and Haugen, A. 1997, Genotypes of glutathione transferase M1 and P1 and their significance for lung DNA adduct levels and cancer risk, *Carcinogenesis* **18,** 1285.

Saeki, Y., Tamura, K., Yamamoto, Y., Hatada, T., Furuyama, J. and Utsunomiya, J. 1997, Germline p53 mutation at codon 133 in a cancer-prone family, *J Mol. Med* **75,** 50.

Schuffenecker, I., Virally-Monod, M., Brohet, R., Goldgar, D., Conte-Devolx, B., Leclerc, L., Chabre, O., Boneu, A., Caron, J., Houdent, C., Modigliani, E., Rohmer, V., Schlumberger, M., Eng, C., Guillausseau, P.J. and Lenoir, G.M. 1998, Risk and penetrance of primary hyperparathyroidism in multiple endocrine neoplasia type 2A families with mutations at codon 634 of the RET proto-oncogene. Groupe D'etude des Tumeurs a Calcitonine, *J Clin Endocrinol Metab.* **83,** 487.

Seidegard, J., Pero, R.W., Markowitz, M.M., Roush, G., Miller, D.G. and Beattie, E.J. 1990, Isoenzyme(s) of glutathione transferase (class Mu) as a marker for the susceptibility to lung cancer: a follow up study, *Carcinogenesis* **11,** 33.

Shen, C.Y., Lo, Y.L., Chang, S.F., Tseng, S.L., Yu, J.C., Yue, C.T. and Wu, C.W. 1998, Allelic loss of the BRCA1 and BRCA2 genes and other regions on 17q and 13q in breast cancer among women from Taiwan-the area of low incidence but earlier onset, *Proc. Am. Assoc. Cancer Res.* **39,** 338.

Shen, D., Subbarao, M., Chillar, R. and Vadgama, J.V. 1998, Different patterns of germline BRCA1 gene mutations detected in the young minority breast cancer patients, *Proc. Am. Assoc. Cancer Res.* **39,** 181.

Shields, P.G., Bowman, E.D., Harrington, A.M., Doan, V.T. and Weston, A. 1993, Polycyclic aromatic hydrocarbon-DNA adducts in human lung and cancer susceptibility genes, *Cancer Res* **53,** 3486.

Silva, J.M., Gonzalez, R., Gomendio, B., Munoz, G., Provencio, M., Garcia, J.M., Carretero, L., Espana, P. and Bonilla, F. 1998, Allelic losses in the BRCA1 and BRCA2 regions and high malignancy in breast carcinomas, *Proc. Am. Assoc. Cancer Res.* **39,** 339.

Struewing, J.P., Hartge, P., Wacholder, S., Baker, S.M., Berlin, M., McAdams, M., Timmerman, M.M., Brody, L.C. and Tucker, M.A. 1997, The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews, *N. Engl. J Med* **336,** 1401.

Sugimura, H., Hamada, G.S., Suzuki, I., Iwase, T., Kiyokawa, E., Kino, I. and Tsugane, S. 1995,

CYP1A1 and CYP2E1 polymorphism and lung cancer, case-control study in Rio de Janeiro, Brazil, *Pharmacogenetics.* **5 Spec No**, S145.

Szabo, C.I. and King, M.C. 1995, Inherited breast and ovarian cancer, *Hum Mol. Genet* **4 Spec No,** 1811.

Taioli, E., Crofts, F., Trachman, J., Demopoulos, R., Toniolo, P. and Garte, S.J. 1995a, A specific African-American CYP1A1 polymorphism is associated with adenocarcinoma of the lung, *Cancer Res* **55,** 472.

Taioli, E., Trachman, J., Chen, X., Toniolo, P. and Garte, S.J. 1995b, A CYP1A1 restriction fragment length polymorphism is associated with breast cancer in African-American women, *Cancer Res* **55,** 3757.

Taioli, E. and Garte, S.J. 1996, Re: S. J. London et al., Lung cancer risk in African-Americans in relation to a race-specific CYP1A1 polymorphism. Cancer Res., 55: 6035-6037, 1995 [letter; comment], *Cancer Res* **56,** 4275.

Tefre, T., Ryberg, D., Haugen, A., Nebert, D.W., Skaug, V., Brogger, A. and Borresen, A.L. 1991, Human CYP1A1 (cytochrome P1450) gene: lack of association between the Msp I restriction fragment length polymorphism and incidence of lung cancer in a Norwegian population, *Pharmacogen* **1,** 20.

Topinka, J., Binkova, B., Mrackova, G., Stavkova, Z., Benes, I., Dejmek, J., Lenicek, J. and Sram, R.J. 1997, DNA adducts in human placenta as related to air pollution and to GSTM1 genotype, *Mutat. Res* **390,** 59.

Uchino, S., Noguchi, S., Adachi, M., Sato, M., Yamashita, H., Watanabe, S., Murakami, T., Toda, M. and Murakami, N. 1998, Novel point mutations and allele loss at the RET locus in sporadic medullary thyroid carcinomas, *Jpn. J Cancer Res* **89,** 411.

van der Luijt, R.B., Meera Khan, P., Vasen, H.F., Breukel, C., Tops, C.M., Scott, R.J. and Fodde, R. 1996, Germline mutations in the 3' part of APC exon 15 do not result in truncated proteins and are associated with attenuated adenomatous polyposis coli, *Hum Genet* **98,** 727.

Varley, J.M., McGown, G., Thorncroft, M., Cochrane, S., Morrison, P., Woll, P., Kelsey, A.M., Mitchell, E.L., Boyle, J., Birch, J.M. and Evans, D.G. 1996, A previously undescribed mutation within the tetramerisation domain of TP53 in a family with Li-Fraumeni syndrome, *Oncogene* **12,** 2437.

Vaury, C., Laine, R., Noguiez, P., de Coppet, P., Jaulin, C., Praz, F., Pompon, D. and Amor-Gueret, M. 1995, Human glutathione S-transferase M1 null genotype is associated with a high inducibility of cytochrome P450 1A1 gene transcription, *Cancer Res* **55,** 5520.

Vineis, P., Caporaso, N., Tannenbaum, S.R., Skipper, P.L., Glogowski, J., Bartsch, H., Coda, M.,

Talaska, G. and Kadlubar, F. 1990, Acetylation phenotype, carcinogen-hemoglobin adducts, and cigarette smoking, *Cancer Res.* **50,** 3002.

Vineis, P., Bartsch, H., Caporaso, N., Harrington, A.M., Kadlubar, F.F., Landi, M.T., Malaveille, C., Shields, P.G., Skipper, P., Talaska, G. and et al, 1994, Genetically based N-acetyltransferase metabolic polymorphism and low-level environmental exposure to carcinogens, *Nature* **369,** 154.

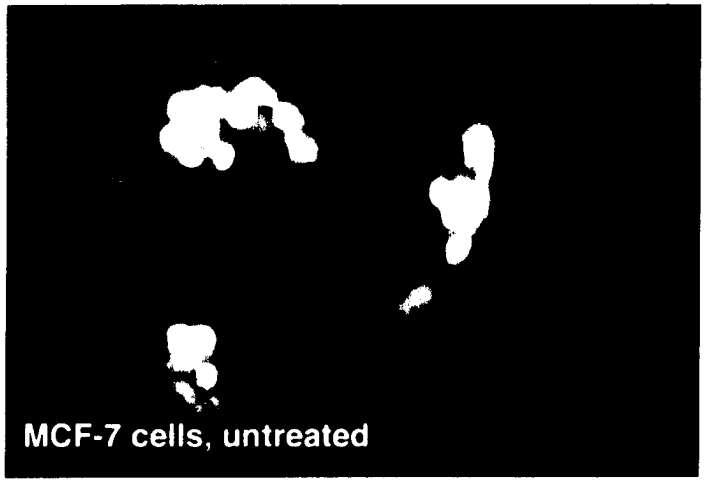
Wedlund, P.J., Kimura, S., Gonzalez, F.J. and Nebert, D.W. 1994, 1462V mutation in the human CYP1A1 gene: lack of correlation with either the Msp I 1.9 kb (M2) allele or CYP1A1 inducibility in a three-generation family of east Mediterranean descent, *Pharmacogenetics.* **4,** 21.

Wijnen, J., Khan, P.M., Vasen, H., Menko, F., van der Klift, H., van den Broek, M., van Leeuwen-Cornelisse, I., Nagengast, F., Meijers-Heijboer, E.J., Lindhout, D., Griffioen, G., Cats, A., Kleibeuker, J., Varesco, L., Bertario, L., Bisgaard, M.L., Mohr, J., Kolodner, R. and Fodde, R. 1996, Majority of hMLH1 mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15-16, *Am. J Hum Genet* **58,** 300.

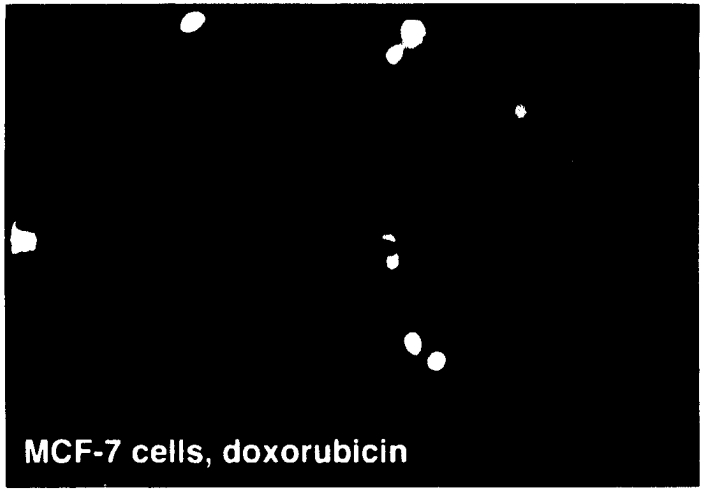
Zedenius, J., Larsson, C., Bergholm, U., Bovee, J., Svensson, A., Hallengren, B., Grimelius, L., Backdahl, M., Weber, G. and Wallin, G. 1995, Mutations of codon 918 in the RET proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas, *J Clin Endocrinol Metab.* **80,** 3088.

Zhang, Z.Y., Fasco, M.J., Huang, L., Guengerich, F.P. and Kaminsky, L.S. 1996, Characterization of purified human recombinant cytochrome P4501A1-Ile462 and -Val462: assessment of a role for the rare allele in carcinogenesis, *Cancer Res* **56,** 3926.

Zhong, S., Howie, A.F., Ketterer, B., Taylor, J., Hayes, J.D., Beckett, G.J., Wathen, C.G., Wolf, C.R. and Spurr, N.K. 1991, Glutathione S-transferase mu locus: use of genotyping and phenotyping assays to assess association with lung cancer susceptibility, *Carcinogenesis* **12,** 1533.


MCF-7 cells, untreated


T47D cells, untreated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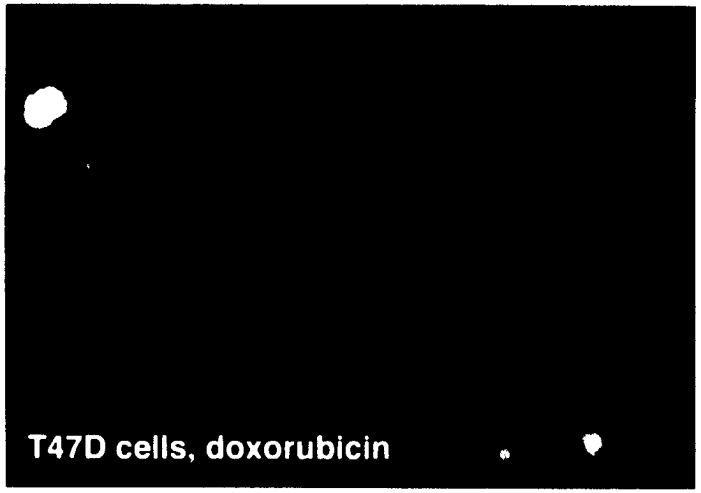
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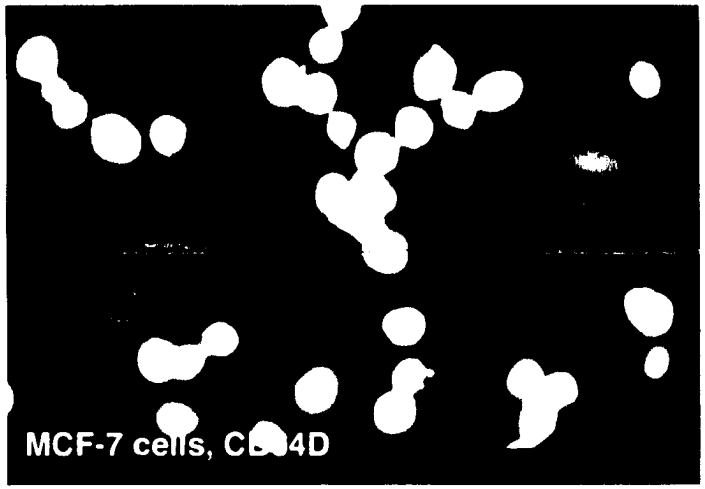
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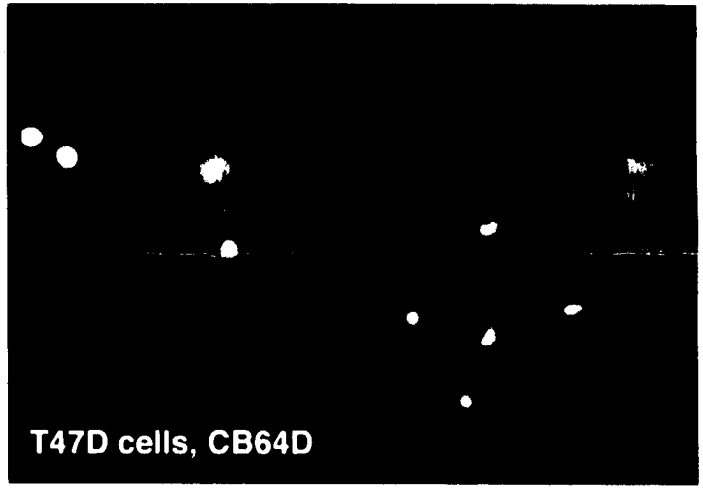
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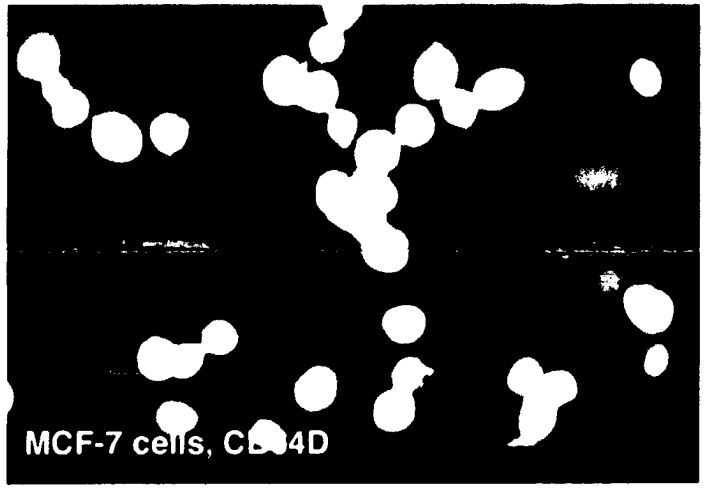
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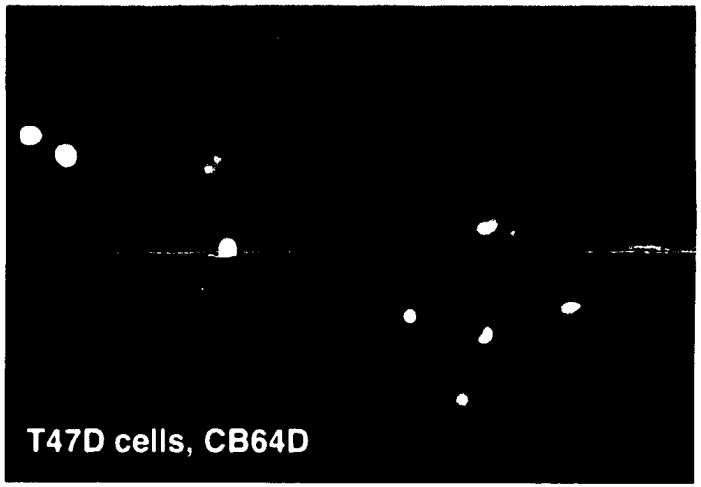
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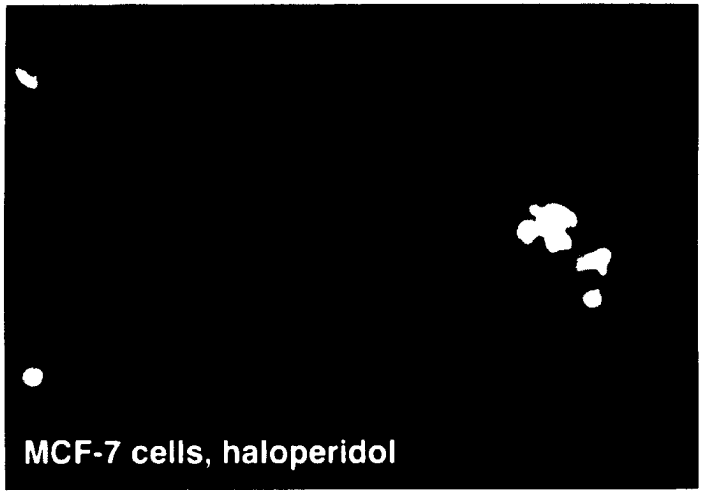
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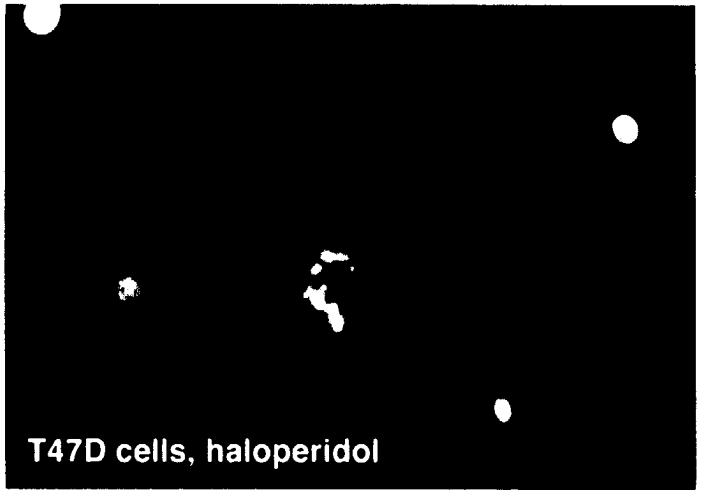
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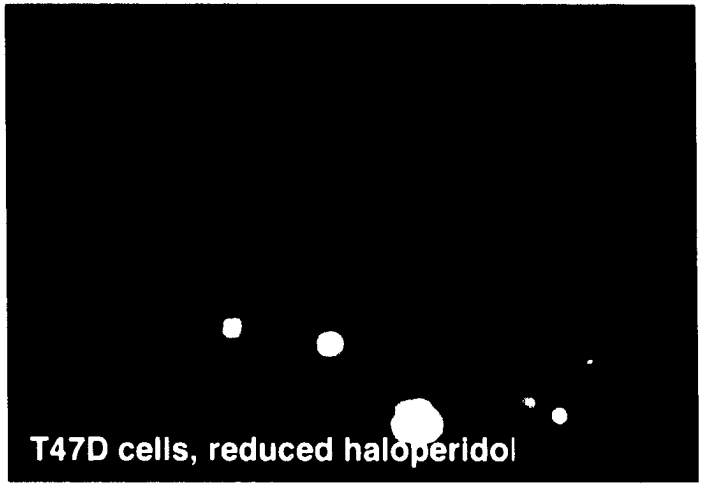
MCF-7 cells, reduced haloperidol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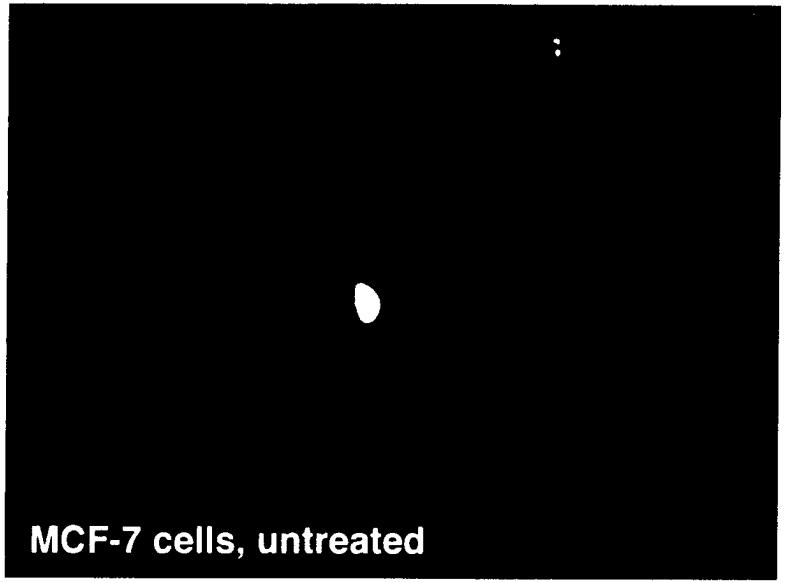
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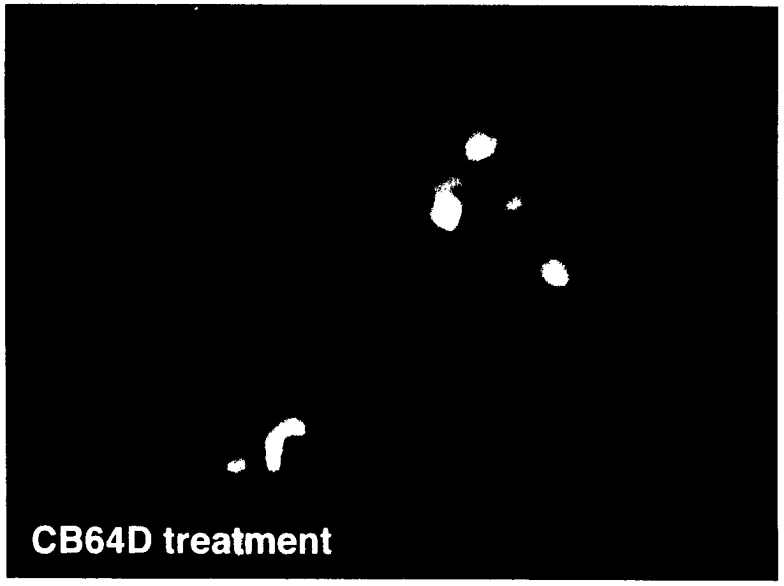
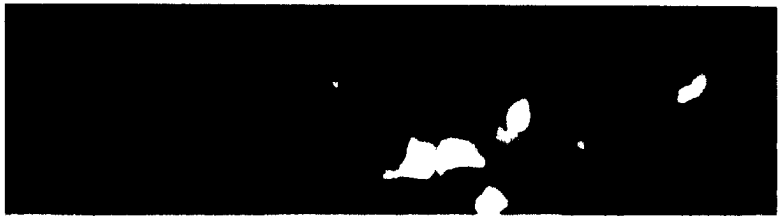
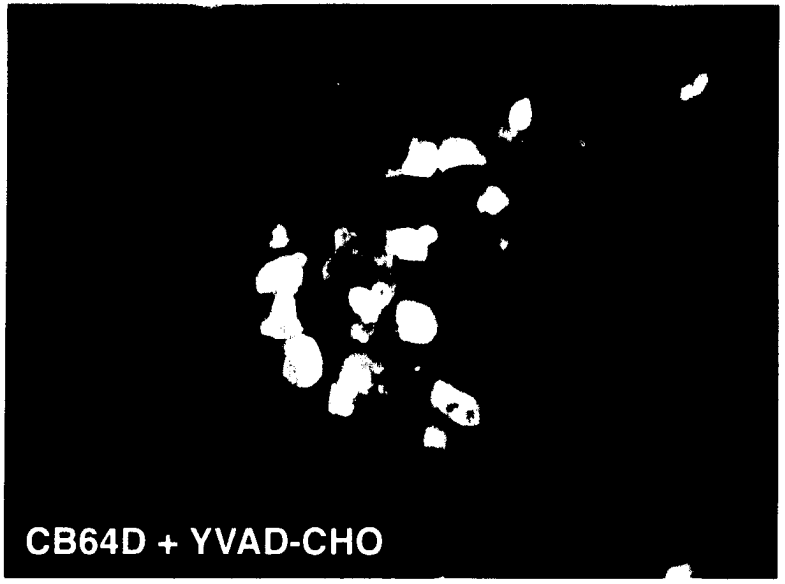

CB64D treatment




CB64D treatment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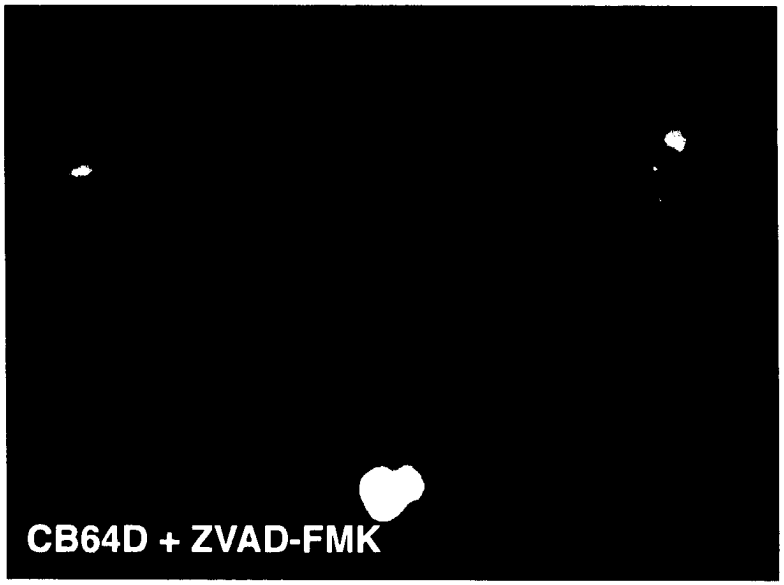
CB64D + ZVAD-FMK