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PRINCIPAL INVESTIGATOR: Dr. Marti Jett

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13. ABSTRACT *(Maximum 200 words)*
MCF-7 wild type(WT) cells have been examined for their sensitivity to various inhibitors of arachidonate metabolism as outlined in the proposal. One aim was to determine the effects of cyclooxygenase(CO) vs lipoxygenase(LO) inhibitors on proliferation in breast cancer cultures. Optimal supression of proliferation was observed using inhibitors of 5-LO, platelet activating factor, and protein kinase C. CO inhibitors stimulated proliferation at conc. specific for PGH synthase-1. New drugs, heteropoly anions, free-radical scavengers which probably alter arachidonate metabolism, are quite effective antiproliferative agents. In order to see if these inhibitors have potential in chemotherapy, preliminary experiments have established their relative lack of toxicity in cultures of normal human bone moarrow stroma cells. Certain of the heteropoly anions were not toxic in bone marrow cultures at concentrations showing effective antiproliferative activity in MCF-7 WT and multidrug-resistant (MDR) cells; these inhibitors did not readily induce development of MDR in WT cultures. Experiments have been done to establish quiescence in cultures; these cultures recover from quiescence to respond normally to signals. Quiescent cultures are being examined for agonist-stimulated arachidonate metabolism

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ARACHIDONATE METABOLISM IN BREAST CANCER CULTURES: IDENTIFICATION OF ANTOAGNISTS/ AGONIST FOR POSSIBLE INTERVENTION STRATEGIES.

PRINCIPAL INVESTIGATOR: Marti Jett, Ph.D.

INTRODUCTION:

Arachidonic acid metabolism.

This research proposal was prompted by the publication of epidemological data suggesting that people who took low dose aspirin daily for heart disease had a decreased incidence of colon cancer relative to a control group {26}. It was not clear if these individuals had also changed their food habits and life style or if other factors might also have contributed to the observed results. This publication brought out the fact that we had little understanding of the role of arachidonate metabolites in cancer in general and in breast cancer, in particular. Many of the arachidonate metabolites have been characterized as initiating cascades of other biologically active molecules such as cytokines, activation of kinases and calcium mobilization; these activators of cellular function are explosive and produce long lasting alterations in cellular functions.

i. Release of arachidonic acid from phospholipids. Arachidonic acid is released from the Sn-2 position of phospholipids by the family of enzymes phospholipasTe A2 (PLA2). The various phospholipase A2's have different specificity toward phospholipid headgroups (choline, ethanolamine, inositol, etc.) as well as to the Sn-2 fatty acid. PLA2's are usually most efficient with polyunsaturated fatty acids in the Sn-2 position, although certain commercial enzymes (porcine pancreas PLA2) are most effective with oleoyl(18:1)-phosphatidylethanolamine {17}. In our prievious work with MCF-7 WT and drug-resistant cells as well as with N4TG1 , neuroblastoma cells, we observed a very active PLA2 activity with specificity toward either linoleoyl, or arachidonyl phosphatidylinositol {10-19}.

ii. Metabolism of arachidonic acid. A simplified chart of arachidonic acid metabolism is shown in Fig. 1. The two major classes of enzymes responsible for arachidonic acid metabolism are the cyclo-oxygenases and lipoxygenases. Cyclo-oxygenases produce the prostanoids and thromboxanes. The best known of these is probably prostaglandin E2, the compound involved in pain and inflamation. Other prostanoids have been implicated in cell proliferation. A multidrug-resistant breast cancer cell line{4,5}, MCF-7 ADR^{10}, has huge increases in prostacyclin H synthetase {28}. Lipoxygenases alter arachidonic acid by first adding a hydroperoxy group at a specific carbon position, and the resulting compounds are named hydroperoxyeicosatetraenoic acids (HPETE). The second generation of compounds are hydroxyeicosatetraenoic acids (HETE's). The lipoxygenases are named for the carbon position which they modify; the 5, 12, and 15 being the most common. The 12- and 15-HETE's have been shown to stimulate cell proliferation in low concentrations {1-3}, however, they become quite toxic at higher concentrations. The 5-lipoxygenase (LO) pathway has been studied extensively because

of its involvement in acute allergy symptoms, shock, adult respiratory distress syndrome{2}. We have identified a 5-LO metabolite, 5-HETE, as a possible growth (co)factor in small cell lung carcinoma and now, as will be described, have implicated it in breast cancer. This compound is also converted to lipoxins, metabolites characterized to stimulate protein kinase C. Lipoxins are abundantly produced in response to growth factors in the tumor cell systems which we are studying. In addition, specifically hydroxylated linoleic acid has been shown to occur upon stimulation of 3T3 cells with EGF {8}. It is unclear if the same lipoxygenase utilize both arachidonic and linoleic acids.

iii. Inhibitors of arachidonate metabolism. We have used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects. Indomethacin (cyclo-oxygenase inhibitor), eicosatetrayenoic acid, ETYA (a structural analog of arachidonic acid which blocks both cyclo-oxygenase and lipoxygenase pathways), nordiguardaic acid (lipoxygenase inhibitor), and MK591, AA861 (5-lipoxygenase inhibitors). Several of the 5-lipoxygenase inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis {1-3, 11}. We have used some of these in studies involving toxins, etc and in the results section, we now we describe our studies with MCF-7 WT breast cancer cells.

iv) In previous studies from our laboratory, we observed that IGF-I stimulated production of bioactive arachidonate metabolites in NCI-H209 small cell lung carcinoma cultures (collaborative study with J.L. Mulshine and I. Avis, Biomarkers and Prevention Research Branch, NCI, manuscript accepted, J. Clin. Invest.). In that study we found that (a) 5-LO products were overproduced (b) in quiescent cultures, 5-HETE stimulated cell proliferation, (c) inhibitors of 5-LO metabolism blocked proliferation, (d) the 5-LO enzymes were over-expressed in response to IGF-I. In this report we will present preliminary evidence that similar pathways are crucially involved in proliferation in cultures of MCF-7 WT breast cancer cells.

v) Study of AA861 inhibition of proliferation in the breast cancer cell line, MCF-7 WT. MCF-7 WT cell proliferation {6} was completely blocked by the 5-lipoxygenase, AA861 (collaborative studies with R.L. Fine, Duke/Va Medical Center, Durham, NC). Synchronized cells held by thymidine block in G_0, when released from the block, took 2-3 hr to develop cell surface markers typical of the G_S phase, and arachidonate analysis from 15min to 5 hr showed that 5-HETE production increased rapidly upon washing out the block and continued to accumulate for about 90 minutes. As 5-HETE production fell, an elevation was seen in the 5-HPETE and 15-HETE metabolites. Interestingly, asynchronous control cultures displayed increased 5-HETE production upon fluid change, although the time of production was delayed and the quantity produced was markedly less. We have proposed that 5-HETE has characteristics of a growth (co)factor.

MATERIALS:

Materials: ITS (insulin, selenium, transferrin; Sigma Chemical Co, St. Louis, MO); IMEM (improved minimal essential medium; BioFluids, Walkersville, MD); BSA (bovine serum albumin; Sigma Chemical Co, St. Louis, MO); complete medium (IMEM, 7% fetal bovine serum, 1% MEM vitamins and 1% antibiotics; all from BioFluids, Walkersville, MD); bioactive lipid standards and many specific inhibitors were purchased from BioMol Inc., Plymouth Meeting, PA.

Cell cultures: MCF-7 WT cells were grown in phenol red-free IMEM to avoid problems with estrogen-like activities of phenol red. Cells were subcultured twice per week to prevent irreversible clumping. Cultures were discontinued after 15 subcultures and new cultures brought up from frozen stock. Cultures were tested weekly for estrogen receptor status. We have used these cultured cells for approximately 8 years.

METHODS:

1. Establish conditions for developing quiescence in cultures of MCF-7 cells. This is information will establish the protocol to use as the model for studying generation of bioactive lipids in response to growth factors +/- specific enzyme inhibitors; additionally, the protocol will be used to verify the inverse hypothesis, that the identified bioactive compounds are crucial for proliferation in breast cancer cultures. Conditions which produced the following results will be selected: i) cells do not detach from the matrix, ii) cells have a decreased proliferation rate after 2 days culture in limited nutirents, and iii) cells recover from the limited nutrient period and show proliferation both by thymidine incorporation and by microscopically determining the number of viable cells.

a) Day 1: Plate cells at a density which will permit them to remain in culture 1 week (10,000 /2 sq cm. Plate in complete medium containing 7% fetal bovine serum and other usual additives. (Serum is necessary for the cells to attach to the plastic dishes).

b) Day 3: Remove the fluid, gently wash the cultures with saline. Replace with serum-free medium containing various additives to determine which options produce the desired results (listed above)

c) Day 4: i) Plate 1. Add ^{3}H[methyl]-thymidine to cultures being evaluated for proliferation by that method.

d) Day 5: i) Plate 1. Harvest thymidine containing cultures; ii) Plate 2. trypsinize cells from designated wells and determine number of viable cells by direct microscopic counting of cells, iii) Plate 3. remove the culture fluid from the duplicate set of cultures and replace with nutrients, growth factors etc (to see if the cultures are still capable of responding to stimuli and undergoing proliferation).

e) Day 8: Evaluate cells for response to nutrients added on day 5.

2. Establish proliferation assays which provide complementary information regarding cell proliferation and growth. Since one aim is to determine inhibition of proliferation, it is necessary to have more than one screening assay since many drugs or inhibitors interefere with proliferation assays. .MCF-7 WT cells grow attached to the plastic matrix, an important point for consideration. We have carried out proliferation assays in our laboratory for the past 12 years; however, the new Top Count scintillation

counter which we are now using is so powerful that we needed to take the time to compare many options and see what labor-saving techniques can be successfully used.

i) Comparisions were made using ^{3}H[methyl]-thymidine incorporation in plates vacuum-harvested from the bottom (filter-bottom plates, Millipore Corp., New Bedford, MA) vs top harvesting (which required trypsinization of the cells to remove them from their attachment to the plastic).

ii) Cytolite assay, a chemiluminescence assay (not requiring trypsinization of the cells to remove them from the matrix) from Packard Chemicals, New London, CT, was used to evaluate proliferation in screening for effective inhibitors of proliferation.

iii) Fluorescence Assay: The ligand becomes fluorescent upon binding to DNA. Therefore, it is not necessary to separate bound/free (as in the case of thymidine), but the cells must be lysed for this assay (Molecular Probes, CA).

iv) Clonogenic Assay is the final verification that cellular proliferation is/is not altered in the presence of drugs/ inhibitors.

3. Screening of inhibitors of the lipoxygenases, cyclo-oxygenases, and other bioactive lipids to identify those which have antiproliferative activity at acceptable concentrations. The methods used in these studies are described in the previous section.

4. Synthesis of heteropoly anion free-radical scavengers: Syntheses of high oxidation state manganese-substituted heteropolyanions. We first isolated and characterized Mn^{IV} substituted Keggin polyanions ($[XW_{11}\ Mn^{IV}\ O_{40}]^{n-}$, X = Si, B, and Zn) using X-ray difraction, Extended X-ray absorption fine structure method, magnetic susceptibility, electrochemistry, and routine spectroscopic methods {35}. Di-manganese substituted γ-Keggin polytungstosilicates, γ-$[SiW_{10}\ Mn_2\ O_{40}]^{m-}$, were synthesized{22-25, 29-34} . Oxidation of tetra-manganese substituted polyaions, $[P_2\ W_{18}\ Mn_4\ O_{68} \bullet 2H_2 O]^{10-}$ game two mixed valent compounds were synthesized and characterized {28} as previously described {35-38}. Oxidation of alykene with iodosobenzene (PhIO) was examined using above mono-, di-, and tetra manganese-substituted polyanions in the presence and absence of air. The oxidized products were identified by GC-Mass and comparson the retention time with authentic compounds

5. Determination of inhibition of proliferation in breast cancer cell cultures by heteropoly anion free-radical scavengers:

The techniques described previously have been used for determination of proliferation in breast cancer cell cultures. Heteropolyanions have been shown to have anticancer activities {39-41}

6. Determination of toxicity to cultures of human bone marrow cells by heteropoly anion free-radical scavengers. Studies to determine if there is a reasonable "therapeutic window" of differential toxicity to normal bone marrow cultures *vs* breast cancer cells:

#1. Separation of light-density bone marrow cells. Human bone marrow will be obtained from donor. Marrow specimen will be diluted 1:1 (vol/vol) in sterile calcium and magnesium-Dulbecco's phosphate-buffered saline (DPBS). Twenty mL of diluted marrow cells will be layered over an equal vilume of ficon gradient solution. Centrifuge the solution at 400g for 30 min. at room temperature. The buoyant nuleated marrow cells will

br collected from gradient interfaces and washed twice by centrifugation with DPBS. The cells will be suspended in Lone-Term Culture Medium (LTCM).
#2. Stroma colony assay was performed in LTCM using methods adapted from Dr. Vincent La Russa and others {42, 7}. Above $2x10^5$ light-density cells were plated in 4 well plates with 5 mL LTCM in the absence or in the presence of heteropolyanions or inhibitors. The cells were incubated at 37C, 5% CO_2, and 100% humidity for two or four weeks. The medium with heteropolyanions or inhibitors were changed every week. The stroma colonies were fixed and staining after two and four weeks. The stained stroma colonies were washed and dried in the air. The total number of colonies were counted.
#3. Colony assay for hematopoietic progenitor cells was carried out in methylcellulose cultures (4). Light-density cells (10^5 cells/plate) were plated in 35-mm-diameter gridded tissue culture plates with 1 mL methylcellulose mediumin the absence or in the presence of heteropolyanions or inhibitors. The methylcellulose medium contains 1.1% methylcellulose in IMDM, 30% (vol/vol) FBS, 2.5 U/mL of human recombinant erythropoietin, and 10% (vol/vol) giant-cell tumor conditioned media. The cultures were incubated at 37C, 5% CO_2, and 100% humidity. The hematopoitic colonies were counted using an inverted phase microscope at 14-21 days. CFU-GM, BFU-E, and CUF-Mix were recognized using standard criteria of colonal morpholgy.
#4. Human bone marrow stroma cells will be cultured for three weeks in LTCM with weekly changing medium. The stroma will be unattached and plated in 35-mm diameter gridded tissue culture plates in LTCM. After 24 h, the medium will be changed to heteropolyanions in LTCM and incubated for a week and then remove the heteropolyanion medium and wash with 1X PBS buffer solution. The untreated human bone marrow light-density cells in methylcellulose (the same as described in Experiment #2 without GCT) will be plated on the top of the treated stroma cells. After the incubation, colony formation was determined using an inverted phase microscope at 14-21 days. CFU-GM, BFU-E, and CUF-Mix were recognized using standard criteria of colonal morpholgy. IC50 values in normal cells vs tumor cells are being compared so that we can select ca. 3 HPA's.

7. Arachidonate metabolism: HPLC separation, identification and quantitation of ca. 30 arachidonate metabolites in a single run was devised by our laboratory {27}. We have established a pattern for determination of the kinetics of formation of even transient arachidonate metabolites in cell cultures +/- agonists or antagonists. The method can be adapted for the separation, and identification of radioactive metabolites from cell cultures, or for separating, identifiying and quantitating miniscule amounts (fMol & pMol) in samples from animals or tissues.

8. Agonist stimulated arachidonate metabolism: IGF-I stimulates proliferation quiescent MCF-7 cultures {43}. The objective of this proposal is to identify bioactive lipids which are being generated in response to growth factor stimulation in the presence and absence of specific arachidonate inhibitors or heteropolyanion treated MCF-7 cells. The MCF-7 cell cultures (an adherent cell line) will be plated in 4-well cluster plates and 24 hr later will be incubated overnight arachidonic acid (approximately 10 uCi/well). We have found this culture pattern to produce metabolites with sufficient radioactivity. The

culture fluid will be removed, a saline wash performed, and buffer added (buffer designed for optimal phospholipase A2 activity). Control and agonist/antagonists will be added and the reaction stopped (acidified) at selected time periods from 15 sec through 2 hr (about 12 different time points for each series (control, agonist, etc.). We usually limit an experiment to approximately 50 wells. The cells will be scraped from the wells, briefly sonicated to insure cell disruption (some arachidonate metabolites may remain intracellular), and centrifuged at 20,000 x g to pellet all insoluble material. The internal standard is then added to the supernatant solution, the pH adjusted, arachidonate metabolites extracted and the samples prepared and run on reverse phase using a C-18 column for HPLC analysis. Platelet activating factor fraction will be separated away from arachidonate metabolites at the extraction step and will be assayed. Meanwhile, the pelleted material will be extracted by Bligh-Dyer procedure and the extracts applied to thin layer platess to run in solvent systems designed to resolve phospholipids or diglycerides/fatty acids. The latter techniques give interesting information when comparing phospholipids present at the beginning of the experiment (samples under 2-3 minutes) with those present at the end of the experiment (>2 hr).

RESULTS AND DISCUSSION:

Experiments to establish quiescence in MCF-7 cell cultures. Cells were plated (complete medium) in 24-well cluster plates and on Day 3 after removal of the initial culture fluid (as described in methods), the following conditions were used for cell cultures: a) no fluid change, b) fluid changed but replaced with the same complete medium used in plating, c) IMEM only, d) IMEM plus ITS, and e) IMEM plus 0.1% BSA. Of these options, IMEM alone was the only condition in which cellular proliferation, as measured by thymidine incorporation, was markedly diminished.

The next part of the question posed was to deterimine if cells could recover from nutrient deprivation and respond to normal culture conditions. Parallel experiments were now carried out in 24 well plates (for thymidine incorporation) and in 6-well plate clusters (to determine actual cells/well). The ITS option (above) was not included. On Day 5, one set of 24-well plates were harvested to determine thymidine incorporation and a set of 6-well plates were used to determine the actual numbers of cells obtained under each condition. The results are shown in Table I. Additionally, on day 5, in parallel cultures, the fluid was changed in all the wells to standard complete medium and they were cultured until Day 8. At that time thymidine incorporation and actual numbers of cells were determined as for day 5 (Table I). The thymidine incorporation in wells containing IMEM alone for two days was increased the most upon harvesting at Day 8. The numbers of cells was increased above those seen on Day 5. Although the other 3 incubation conditions had higher cell counts on Day 5, there division rate slowed by Day 8. This slowing of the proliferation rate is typical of these cells. Because they grow in pyramid-like clusters, we have previously seen that the in fourth or fifth day of culture, the rate of proliferation is significantly diminished relative to days 2-3. Therefore the conditions selected to obtain

quiescence in the cultures was the sequence outined in Methods using a fluid change to IMEM alone for 2 days.

We need to characterize this state further to determine a) the percentage of the cells in the Go stage of division (by immunocytochemistry{}), and b) to verify that IGF-1 or other agonist increases proliferation and cell numbers under the conditions described above (which used complete medium).

Proliferation assays. We have obtained good dose/response results of ^{3}H-[methyl]thymidine using top-harvesting of trypsinized cell cultures (see materials and methods). Bottom harvesting with filter plates (Millipore Corp., MA) was not sufficiently reproducible to use. The chemiluminescence assay was successful for the MCF-7 WT cell line, but not for other breast cancer cell lines which we are also using. The fluorescence assay is still under evaluation. As mentioned in Methods, the Top Count scintillation counter is a significant labor-saving piece of equipment which we are still learning to optimize for a proliferation screening assay. Additionally, the clonogenic assay is used to verify results obtained with the proliferation screening assay.

A typical dose/ response curve is shown in Figure 2. In this study, PCA-4248, a platelet activating factor receptor antagonist, was evaluated for its activity in blocking proliferation in MCF-7 WT breast cancer cultures. Various concentrations were tested from 0-50 μM; for each point shown, 8 replicate wells were averaged and the standard error or the mean determined. This graph shows the results of three individual experiments from different days. From this graph, and similar graphs of other specific inhibitors, the concentration giving 50% inhibition (IC_{50}) was determined. In this case, using the average obtained from the three dose/ response curves shown, the IC_{50} was 13.7 ± 1.9 μM. This is a reasonably non-toxic drug in cultures of normal cells. These concentrations may be therapeutically achievable.

Screening of inhibitors of the lipoxygenases, cyclo-oxygenases, and other bioactive lipids to identify those which have antiproliferative activity at acceptable concentrations. As discussed the the previous section, the IC_{50} was determined for specific inhibitors of arachidonate metabolism in regards to their ability to block proliferation. The list of arachidonate inhibitors in Table II shows the average of three separate experiments, each experiment was performed with replicates of 8. Among the inhibitors which were effective antiproliferative agents was MK591, a blocker of 5-lipoxygenase activating protein (FLAP). We have proposed that 5-HETE, a product of 5-lipoxygenase(LO) activation by FLAP has characteristics of a growth (co)factor in cancer cell cultures. (Please note that a patent has been submitted by us showing that blocking 5-LO activity with a variety of specific inhibitors shows promise as anticancer therapeutic and intervention agents; see addendum.) MK591 is currently in clinical trials for treatment of adult respiratory distress syndrome and is well tolerated *in vivo* at concentrations much higher than needed for shrinking / eliminating tumors using a model of small cell lung carcinoma (Avis, Jett, Boyle & Mulshine, manuscript accepted for publication in J. Clin.

Invest, 1995). We also found that a 5-LO enzymatic inhibitor (in contrast to a FLAP inhibitor), AA861 was an effective antiproliferative agent. We do not have extensive toxicity data to normal cells on that drug, however. Nordihydroguaretic acid (NDGA), a non-specific LO inhibitor of 5-, 12- and 15-LO's, showed blocking of proliferation in the MCF-7 breast cancer cultures. In contrast to the antiproliferative effects of lipoxygenase inhibitors, the cyclooxygenase inhibitor indomethacin, was not effective in the concentration range where its effects are limited to the cyclooxygenase system (0.1 uM) rather than to lipoxygenase inhibition (42 uM). A blocker of both pathways (Curcumin) and phospholipase A2 inhibition (dexamethasone) were both ineffective at usable concentrations. Lipoxygenase products can also be generated by P450; ketoconazole and clofibrate are both inhibitors of this system. We do not yet have conclusive results with these inhibitors.

The bioactive lipids from arachidonate metabolism have stimulatory activities on many other signalling pathways such as protein kinase C (PKC) and tyrosine kinases, and phospholipase C. The interaction among these pathways provides the explosive amplification of signal generation which has been characterized in tumor cell systems. Therefore, we also used inhibitors of those pathways. In general they are quite toxic and some of them are currently in clinical trials as anticancer agents (e.g. staurosporin, a PKC inhibitor). Indeed, the IC_{50} of several of these drugs show the crucial role of these pathways for proliferation of MCF-7 breast cancer cultures. They are difficult therapeutic targets because their inhibitors are also quite toxic to normal cells. Examples are chelerythine, sphingosine, thapsagargin, and K-252. These will be tested in bone marrow cultures to determine their toxicity.

Determination of inhibition of proliferation in breast cancer cell cultures by heteropoly anion free-radical scavengers synthesized in our laboratory by Dr. XiaoYan Zhang (NRC fellow): The structure of typical heteropoly anion (HPA) free-radical scavengers is shown in Figures 3 & 4. The list of HPA's synthesized and evaluated in the MCF-7 WT breast cancer cell cultures is shown in Table III. Our initial data suggest that these stable compounds interfere with arachidonate metabolism and inhibit nitric oxide generation. We currently do not have extensive toxicity data to normal cells, but are proceeding with that study. Until we know relative toxicity, we cannot make informed judgments of which HPA's might be useful for continued studies in blocking bioactive lipid generation and interfering in signal cascades. One promising HPA (#5) was effective at low concentrations in blocking proliferation in breast cancer cultures and we have preliminary data showing lack of toxicity to normal human bone marrow cultures (see the following section).

Determination of toxicity to cultures of human bone marrow cells by heteropoly anion free-radical scavengers. Studies to determine if there is a reasonable "therapeutic window" of differential toxicity to normal bone marrow cultures *vs* breast cancer cells. Preliminary studies using human bone marrow stroma cells show that two of the three compounds tested did not block colony formation in these cultures (Table

IV). Additional concentrations must be tested and other bone marrow assays performed to gain information regarding toxicity of these drugs.

FUTURE DIRECTIONS AND GOALS:

1. The most urgent objective is to complete the quiescence experiments and establish defined conditions for signalling studies. Our current data indicate that culture in IMEM alone meets the conditions regarding a reversible slowing of growth. The questions to be answered relate to characterization of the cells with regard to estrogen receptors, markers of Go, and responsiveness to hormones and other growth factors such as oxytocin and IGF-I.

2. Generation of arachidonate metabolites in response to IGF-I (in quiescent breast cancer cell cultures) is the major thrust of this proposal. In this study, the kinetics of arachidonate metabolite generation will be observed in control and IGF-I stimulated cultures. We will follow the generation and utilization of up to 30 metabolites in an effort to understand the pathways responsible for generation of bioactive lipid intermediates.

3. Alterations is generation of bioactive lipids in the presence of identified inhibitors will be used to confirm our hypothesis that preventing the formation of certain of these compounds will disrupt growth of breast cancer cells which are so dependent on these signalling cascades. These studies will be carried out in quiescent cultures, preincubated for 30 min with the appropriate inhibitor(s), in control and IGF-I stimulated cultures.

4. Normal human bone marrow cultures will be used to determine toxicity to promising HPA's and specific enzyme inhibitors.

Please note that the goals for years 1 and 2 in the initial proposal have been reversed in our studies, because the NRC fellow was unable to start until April. We have carried out the work so far using student and contract hires. We now have a graduate student (Youtong You, from Catholic University) whose thesis work is a portion of the work outlined in this grant. She, and the other student and contract hires involved in this project are focused, dedicated and hard-working. We expect to be able to submit 2 presentations to the American Society for Cancer Research annual meeting, March 96 in Washington, DC.

ACCOMPLISHMENTS:

Patent submitted: USE OF LIPOXYGENASE INHIBITORS AS ANTICANCER THERAPEUTIC AND INTERVENTION AGENTS. James Mulshine and Marti Jett. CIP submitted Mar 14, 1995. Pending.

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TABLE I

ESTABLISHMENT OF QUIESCENCE AND RECOVERY FROM NUTRIENT DEPRIVATION IN CULTURES OF MCF-7 WT BREAST CANCER CELLS

CULTURE CONDITIONS	^{3}H-[methyl]thymidine)[a] Day 5	^{3}H-[methyl] thymidine) Day 8
Fluid changed w/complete medium Day 3[b].	15,227 ± 564	19,543 ± 1187
IMEM alone Day 3.	9,873 ± 802	17,648 ± 938[c]
IMEM containing 0.1% BSA Day 3.	13,731 ± 593	23,342 ± 1579
No fluid change Day 3.	16,387 ± 476	16,781 ± 726

[a] ^{3}H-[methyl] thymidine was added to the cultures for the final 18 hr, the cells harvested by one of the methods listed under "Methods" and the radioactivity incorporated into cells was determined. The data shown are the average of 6 replicates ± standard error of the mean.

[b] Replicate wells

[c] In control wells which did not have fluid changed with complete medium on day 5, approximately 3000 dpm was observed.

TABLE II

INHIBITION OF PROLIFERATION IN CULTURES OF MCF-7 WT BREAST CANCER CELLS BY SPECIFIC BLOCKERS OF BIOACTIVE LIPID GENERATION.

INHIBITOR	DESCRIPTION OF ACTIVITY BLOCKED	IC_{50} = μM[a]
MK571	Leukotriene D4 receptor antagonist	35
MK591	5-lipoxygenase activating protein (FLAP[b]) inhibitor (2nd generation drug at FDA)	18 ± 0.9
MK886	5-lipoxygenase activating protein (FLAP) inhibitor (1st generation drug at FDA)	23.4 ± 2.1
Nordihydroguaiaretic acid (NDGA)	5-, 12-, 15-lipoxygenase inhibitor, P-450 inhibitor, free-radical scavenger	37.2
CHELERYTHINE CHLORIDE	Protein kinase C inhibitor	8.1 ± 0.2
INDOMETHACIN	Cyclooxygenase (0.1 μM), lipoxygenase inhibitor (100μM), PGH synthetase inhibitor.	26
KETOCONAZOLE	P-450 inhibitor, 5-lipoxygenase inhibitor	95
SPHINGOSINE	Protein kinase C inhibitor	13.2 ± 0.6
CURCUMIN	Dual inhibitor of 5-lipoxygenase (8 μM) and cyclooxygenase (52 μM) pathways	60
PCA-4248	Inhibitor of platelet activating factor binding to its receptor	13.7 ± 0.9
AA861	5-lipoxygenase enzymatic inhibitor	14
THAPSAGARGIN	Induces the release of calcium from intracellular stores independent of INS(1,4,5)P3 (30 nM).	0.05
K-252	Inhibits protein kinases A & C (0.02 μM), potent inhibitor of trk receptor tyrosine kinases and MAP kinase.	0.2
DEXAMETHASONE	Induces lipocortin (a phospholipase A2 inhibitor), blocks the induction of nitric oxide synthase.	no inhibition
CLOFIBRATE	Induces P-450 catalysis of omega-oxidation of fatty acids and prostaglandins.	50

[a] IC_{50} = μM concentrations, determined by incorporation of ^{3}H-[methyl] thymidine).

[b] FLAP has been shown to function by serving as a membrane bound anchor for the enzyme 5-lipoxygenase; the enzyme is not thought to be active until it translocates to the membrane and becomes complexed to FLAP.

TABLE III

INHIBITION OF PROLIFERATION IN CULTURES OF MCF-7 WT BREAST CANCER CELLS USING POLYOXOTUNGSTATES[a]

Name of polyoxotungstate[b]	IC_{50}(Exp.1)	IC_{50}(Exp.#2)
$K_{12.5}Na_{1.5}[NaP_5W_{30}O_{110}]\bullet nH_2O$	3.8	3.5
$K_{13}[CaP_5W_{30}O_{110}]\bullet nH_2O$	4.5	5.0
$K_{12}[BiP_5W_{30}O_{110}]\bullet nH_2O$	N/A	5.0
$K_{12}[CeP_5W_{30}O_{110}]\bullet nH_2O$	4.3	5.0
$K_{12}[SmP_5W_{30}O_{110}]\bullet nH_2O$	0.5	2.5
$K_{12}[EuP_5W_{30}O_{110}]\bullet nH_2O$	N/A	4.5
$K_{12}[GdP_5W_{30}O_{110}]\bullet nH_2O$	2.0	8.0
$K_{12}[TbP_5W_{30}O_{110}]\bullet nH_2O$	1.0	8.0
$K_{12}[DyP_5W_{30}O_{110}]\bullet nH_2O$	4.3	5.0
$K_{12}[HoP_5W_{30}O_{110}]\bullet nH_2O$	3.0	4.5
$K_{12}[ErP_5W_{30}O_{110}]\bullet nH_2O$	4.3	6.0
$K_{12}[TmP_5W_{30}O_{110}]\bullet nH_2O$	0.25	10
$K_{12}[YbP_5W_{30}O_{110}]\bullet nH_2O$	3.8	3.7
$K_{12}[LuP_5W_{30}O_{110}]\bullet nH_2O$	1.0	5.0
$K_{12}[UP_5W_{30}O_{110}]\bullet nH_2O$	1.0	10

[a] For structures of an example of heteropolyanions, see figures 3 & 4.

[b] n = ranges from 20 to 30.

TABLE IV

STROMA COLONY ASSAY WITH 2 X 10^5 CELLS/WELL IN LONG-TERM CULTURE MEDIUM

Compound (Concentration)	Assay#1	Assay#2	Assay#3	Assay#4	Average
Control	33	27	35		32
$K_{12}[SmP_5W_{30}O_{110}]\bullet nH_2O$(0.1μM)	35	39	36	30	35
$K_{12}[SmP_5W_{30}O_{110}]\bullet nH_2O$(1.0μM)	38	34	46	34	37
Sphingosine (10 μM)	0	0	0	0	0
Sphingosine (30 μM)	0	0	0	0	0
Thapsigargin (1.0 nM)	35	40	36		37
Thapsigargin (10 nM)	35	35			35

LEGENDS TO FIGURES

Figure 1. A schematic diagram showing some of the major bioactive lipid metabolites of arachidonic acid. In parenthesis are names of a few of the inhibitors used in the study; they are placed at the exzymatic step which they are thought to block.

Figure 2. Dose/ response plot of data showing the inhibition of proliferation by a specific blocker of platelet activating factor receptors. IC_{50} was calculated from the three replicate sets of data which are plotted in this graph. Table II shows a list of various inhibitors used, their biological site of action and their IC_{50} in cultures of MCF-7 WT breast cancer cells.

Figures 3 & 4. Three dimensional structural representations of heteropoly anions. These free-radical scavengers are effective antiproliferative agents in cultures of MCF-7 WT breast cancer cells. A list of the heteropoly anions evaluated in this system is shown in Table III. Some of these have been tested for toxicity to normal human bone marrow cultures as well (Table IV).

FIGURE 1

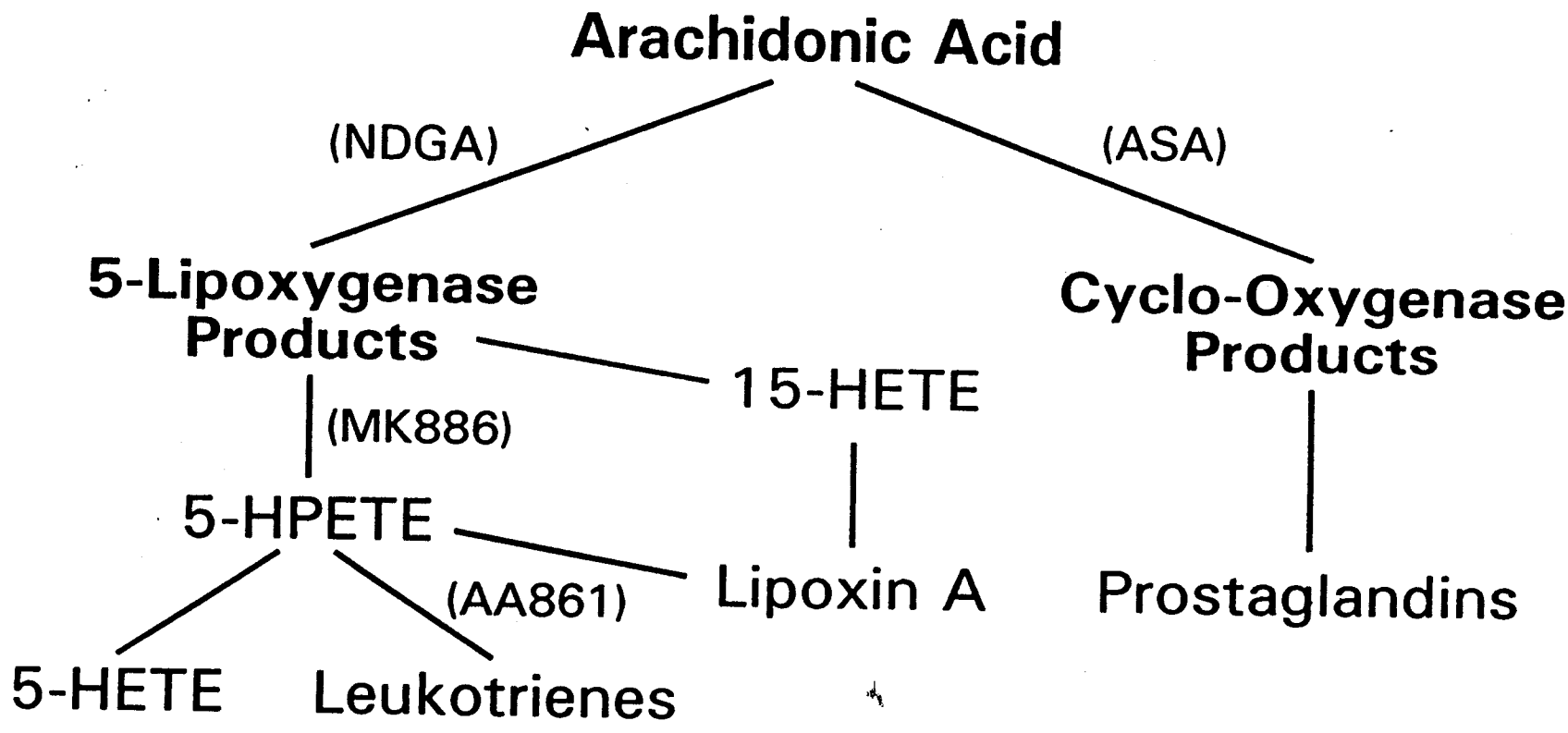

Arachidonic Acid
(NDGA)
(ASA)
5-Lipoxygenase Products
Cyclo-Oxygenase Products
15-HETE
(MK886)
5-HPETE
Lipoxin A
Prostaglandins
(AA861)
5-HETE
Leukotrienes

FIGURE 2

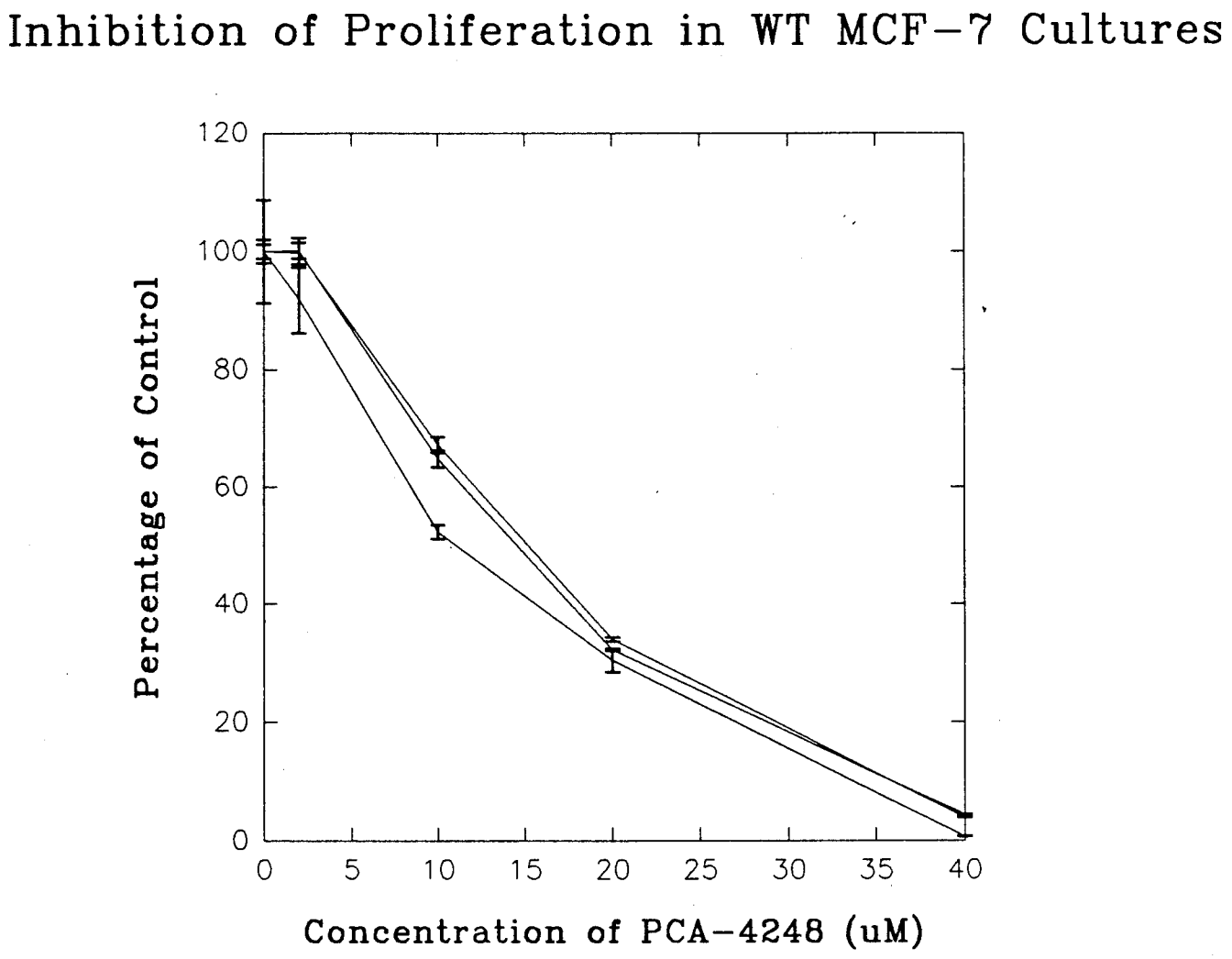

Inhibition of Proliferation in WT MCF-7 Cultures
Percentage of Control
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Concentration of PCA-4248 (uM)

FIGURE 3

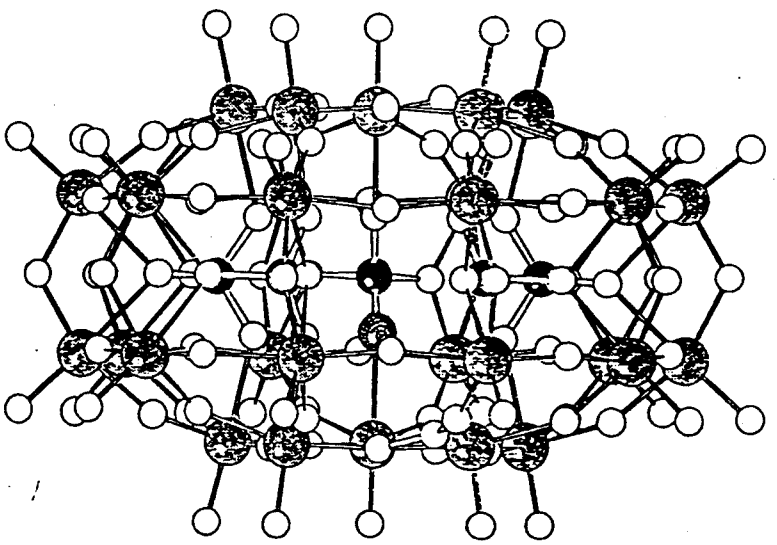
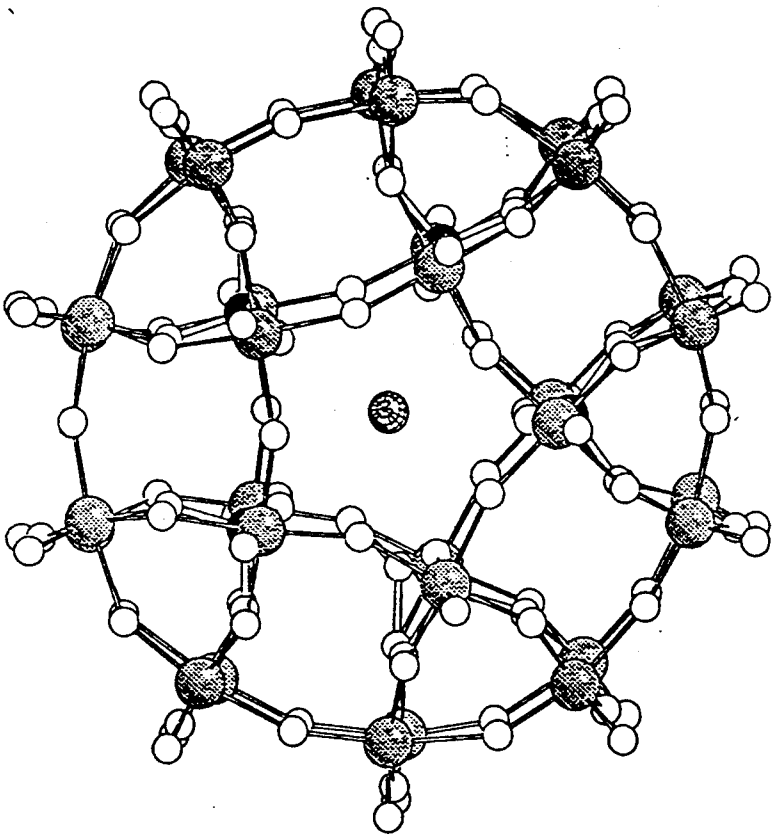


Figure 2. The structure of $[X(PO_4)_5W_{30}O_{90}]^{n-}$, X = Na^+, Ca^{2+}, Bi^{2+}, Ce^{3+}, Sm^{3+}, Eu^{3+}, Gd^{3+}, Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, Yb^{3+}, Lu^{3+}, or U^{3+}.

X: ; W: ; P: ; O:

FIGURE 4



Received 1-28-2000 ack

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REPLY TO
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