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Introduction

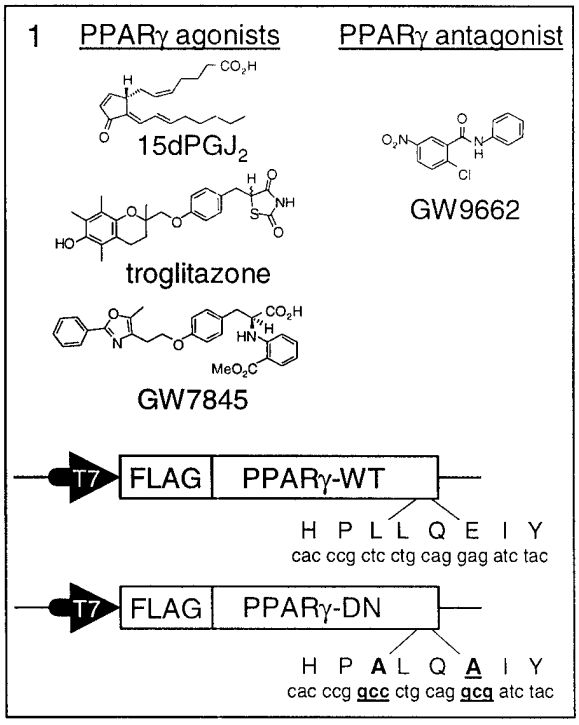
The peroxisome proliferator activated receptor gamma (PPARγ), is a potential therapeutic target for the treatment of breast cancer but the endogenous ligand for PPARγ is not yet known. Recent data suggest that the endogenous ligand of PPARγ may be a bioactive metabolite of arachidonic acid that is synthesized in normal breast tissue. Activation of PPARγ with different agonists (e.g. 15deoxyΔ12,14PGJ_2, troglitazone) elicits different physiological responses in breast cancer cells (i.e. differentiation or apoptosis) raising questions of the role PPARγ plays in normal breast cell physiology. Results from our initial experiments show that prostaglandin metabolites of arachidonic acid inhibit cell cycle progression of MDA-MB-231 breast cancer cells. This cell cycle block induces apoptosis of breast cancer cells and inhibits tumor formation in nude mice. We hypothesize that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPARγ mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPARγ agonists. Understanding the metabolism of fatty acids in breast cancer cells, and elucidating the molecular and signal transduction events that are mediated by PPARγ agonists may lead to novel strategies for the prevention and treatment of breast cancer.

Body

There is extensive literature on the use of retinoic acid and its derivatives, acting through their receptors (RAR and RXR), to arrest or reverse cancer in both animals and humans. Another member of the nuclear receptor superfamily, peroxisome proliferator activated receptor-gamma (PPARγ), has an important role in fat metabolism and adipocyte differentiation. Although its natural ligand is not yet known, synthetic thiazolidinediones, certain fatty acids and metabolites of arachidonic acid, activate PPARγ. Recent data reveal that PPARγ is expressed in colonic tumors and metastatic breast adenocarcinomas, which raises the critical question of its functional significance in human cancers. RXRα and PPARγ agonists together have been shown to induce apoptosis of estrogen receptor positive breast cancer cell lines *in vitro* and attenuate tumor growth in mice. Our studies show that prostaglandin agonists of PPARγ alone inhibit cell cycle progression of both estrogen receptor positive and negative breast cancer cell lines via apoptosis and inhibit tumor formation in nude mice.

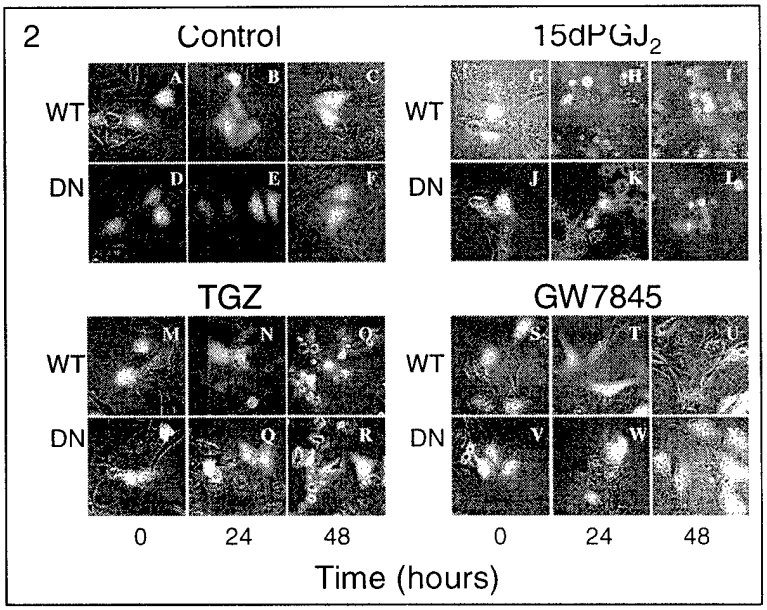
There are three specific aims for the pre-doctoral research hypothesis that human breast cancer cell lines (and human breast cancer tumors) have aberrant PPARγ mediated signal transduction pathways or contain disrupted pathways for the metabolism of fatty acid derivatives that act as PPARγ agonists.

1) The first aim is to determine the physiologic activities of different PPARγ agonists on the proliferation of human breast cancer cell lines and primary human breast cancer cells. We will extend our published findings to include other natural prostanoid and eicosanoid agonists (e.g. PGE_2, DHA), synthetic PPARγ agonists (e.g. BRL49653, ciglitazone) and co-activators that can potentiate the effects of PPARγ agonists (e.g. 9-*cis*-retinoic acid, all-*trans*-retinoic acid).
2) The second aim is to determine the molecular mechanisms and signal transduction events that underlie PPARγ mediated differentiation or apoptosis in breast cancer cells.
3) The third aim is to determine the metabolism of J-series prostaglandins in normal breast tissue and breast cancer cells.



Aim 1: Our studies of other natural and synthetic PPARγ agonists show that several arachidonic acid (AA) metabolites, including 5- and 15-HETEs and 5-and 15-oxo-EETs, are activators of PPARγ. However, of all the naturally occurring metabolites tested, the terminal derivative of prostaglandin D_2 metabolism, 15deoxy$\Delta^{12,14}PGJ_2$ (15dPGJ$_2$), remains the most potent. A major accomplishment of Mr. Clay's was his observation that the published literature cites different physiologic outcomes in various cancer cell lines according to the concentration of PPARγ agonist used. To this end, Mr. Clay authored a review article that documented the differing biological effects of PPARγ activation in diverse cell types (1). Furthermore, Mr. Clay undertook the responsibility of determining if these diverse and opposing biologic outcomes occur in a single cell type (2). However mounting evidence in the literature and in Dr. Chilton's laboratory suggested that the induction of apoptosis might be independent to PPARγ activation. After attending the PPARs Keystone Symposium in February 2000, Mr. Clay was successful in obtaining chemically synthesized selective agonists for each of the PPAR isoforms (α, GW7647; β/δ, GW0742 and γ, GW7845) from GlaxoSmithKlein (GSK) and a dominant negative PPARγ construct (Figure 1). These compounds are 10,000 fold more selective for their respective receptor than for other nuclear receptors. Using a variety of techniques, Mr. Clay has shown that selective activation or inhibition of PPARγ, using the synthetic agonist GW7845 or antagonist GW9662, does not alter cellular proliferation in breast cancer cell lines. Moreover, GW7845 does not induce apoptosis at any concentration tested (Figure 2) (3). Mr. Monjazeb, an MD/PhD candidate in his final year of his dissertation research in Dr. Chilton's laboratory has continued on the studies that Mr. Clay has

completed and will conclude the final year of this pre-doctoral research proposal. Mr. Monjazeb has shown that inhibition of arachidonic acid metabolism blocks cancer cell progression and results in cell death (4).



Aim 2: The molecular mechanisms and signal transduction events that underlie PPARγ mediated differentiation or apoptosis in breast cancer cells are complex and not well understood. Mr. Clay has achieved great milestones in elucidating parts of these pathways. In a screen of 1,176 gene products by cDNA array analysis, Mr. Clay identified particular gene products that are increased in breast cancer cell lines after treatment with $15dPGJ_2$ (5). Of these, the expression of the cyclin dependent kinase inhibitors $p21^{Waf1/Cip1}$ (p21) and $p27^{Kip1}$ (p27) and the cyclins D and E is increased >2 fold. Additionally, the expression of several genes involved in DNA maintenance and repair is decreased >2 fold. Mr. Clay has performed *post hoc* analysis of p21 and p27 expression by Western blot analysis to confirm the results from the cDNA array. Additionally, Mr. Clay has followed up on published reports of the effects of $15dPGJ_2$ in other cell systems to devise a potential mechanism by which $15dPGJ_2$, or other cyclopentenone prostaglandins, may exert such potent anti-neoplastic activity in a variety of cancer cell types (Appendix 1). These studies resulted in a manuscript that was published in *The Journal of Biological Chemistry* (5). Mr. Mojazeb will continue this line of investigation to include other gene products and further elucidate the mechanisms described.

Aim 3: The studies of the metabolism of J-series prostaglandins in normal breast tissue and breast cancer cells are in the beginning stages. Mr. Clay was successful in obtaining a small amount of $[^3H]15dPGJ_2$ through a collaborative effort with Dr. Kirk Maxey of Cayman Chemical. Using $[^3H]15dPGJ_2$ to follow the metabolism of $15dPGJ_2$ in the breast cancer cell line MDA-MB-231, Mr. Clay has noted that after 12 hours, the majority of label is still present as $15dPGJ_2$. In this preliminary study, 66% of $[^3H]15dPGJ_2$ was recovered after 12 hours. The remaining 44% was in the form of more polar metabolites as determined by thin layer chromatography (TLC). These derivative may represent a class of reactive oxygen species (ROS) that further activate PPARγ (Appendix 1). Mr. Clay was unable to determine the structure of these polar metabolites, or their biological activity, due to the limited quantity of material, but Mr. Clay has enlisted the analytical expertise of the laboratory of Dr. Robert Murphy (National Jewish Research Center, Denver, Colorado) to assist with the determination of these structures by negative ion chemical ionization gas chromatography/tandem mass spectrometry (NICI GC/MS/MS). Moreover, Mr. Clay has obtained critical reagents for the study of prostaglandin metabolism. Specifically, Mr. Clay has acquired immuno-reactive antibodies to specific AA metabolizing enzymes. These include antibodies to fatty acid CoA ligase (FACL4), the enzyme that ligates free AA to Co-enzyme A, cyclooxygenase 2 (COX-2), the enzyme which catalyzes the oxidation and cyclization of AA to produce prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) and prostaglandin D_2 synthase (PGDS), the enzyme that catalyzes the formation of PGD_2 from PGG_2/PGH_2. These reagents will be helpful for the investigation of enzymatic levels of these critical metabolizing enzymes. In addition, enzymatic activity assay kits are readily available.

The research completed due to funding from this award has become a springboard for research currently being undertaken in other laboratories at Wake Forest University Baptist School of Medicine and was instrumental in allowing Mr. Clay to obtain a competitive post-doctoral fellowship at Washington University School of Medicine in St. Louis, Missouri. Mr. Monjazeb will make the most of the reagents that Mr. Clay has acquired and the collaborations that Mr. Clay has fostered to continue on the aims outlined in this proposal. Through collaboration with Dr. O'Flahrety (infectious diseases) and Dr. Robbins (radiation oncology) the mechanisms of oxidized lipid intermediates on PPARs on breast cancer cells will be continued to be explored (6,7).

Key Research Accomplishments

- 15deoxy$\Delta^{12,14}PGJ_2$ remains the most potent naturally occurring PPARγ agonist identified.
- The degree of PPARγ activation dictates distinct and opposing biological responses in breast cancer cells, ranging form increased proliferation to differentiation and apoptosis.
- 15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis requires *de novo* expression of critical gene products.
- Dominant negative expression of PPARγ completely abrogates transcriptional activation induced by 15deoxy$\Delta^{12,14}PGJ_2$, but does not rescue breast cancer cells fro 15deoxy$\Delta^{12,14}PGJ_2$-induced apoptosis.
- The mechanism of action of 15deoxy$\Delta^{12,14}PGJ_2$ is not limited to PPARγ activation. 15deoxy$\Delta^{12,14}PGJ_2$ can inhibit isopeptidase activity of the ubiquitin proteosome and inhibit NFκB signaling and can stimulate reactive oxygen species generation. Together, these events lead to induced expression of key gene products that are involved in apoptosis in breast cancer cells.
- 15deoxy$\Delta^{12,14}PGJ_2$ is metabolized to polar derivatives by breast cancer cells.

Reportable Outcomes

• Manuscripts

1. **Clay CE,** Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH, 2000, 15deoxyΔ12,14PGJ_2 induces diverse biological responses via PPARγ activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
2. **Clay CE**, Namen AM, Atsumi G, Trimboli AJ, Fonteh AN, High KP, Chilton FH, 2001, The magnitude of PPARγ activation is associated with important and seemingly opposite biological responses in breast cancer cells. *Journal of Investigative Medicine* 49, 413-420
3. **Clay CE**, Atsumi G, High KP, Chilton FH. (2001) Early *de novo* gene expression is required for 15-Deoxy-$\Delta^{12,14}$ prostaglandin J_2 induced apoptosis in breast cancer cells. *The Journal of Biological Chemistry* 276, 47131-47135
4. Monjazeb A, **Clay CE,** High KP, Chilton FH (2001) Inhibition of arachidonic acid metabolism and remodeling blocks cancer cell proliferation; involvement of PPARgamma. *Prostaglandins and Other Lipid Mediators* 66, 5-12.
5. **Clay CE**, Thorburn J, High KP, Chilton FH. (2002) 15-Deoxy-$\Delta^{12,14}$ prostalgandin J_2-induced apoptosis does not require PPARγ breast cancer cells. *The Journal of Lipid Rresearch* (In press).
6. Zhou W, **Clay CE,** High KP, Chilton FH, Robbins MC. (2002) Cyclopentenone prostaglandins induce apoptosis via oxidative stress in breast cancer cells; Role of lipid and protein oxidation. (in preparation).
7. Rogers LJ, **Clay CE,** High KP, O'Flaherty J. (2002) 5-oxo-EET is a natural PPARγ ligand and blocks proliferation of breast and prostate cancer cells. (In preparation)

Clay CE, Namen AM, Atsumi G, Willingham MC, High KP, Kute TE, Trimboli AJ, Fonteh AN, Dawson PA, Chilton FH. (1999) Influence of J Series Prostaglandins on Apoptosis and Tumorigenesis of Breast Cancer Cells. *Carcinogenesis* 20:1905-1911 (published prior to DOD funding).

• Abstracts

1. *PPARγinduced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* FASEB: Receptors and Signal Transduction, Copper Mountain, CO July 2-9, 2000

2. *15deoxy$\Delta^{12,14}PGJ_2$ inhibits breast cancer cell proliferation via PPARγactivation.* International Society for Preventive Oncology, 5th International Meeting, Geneva, Switzerland, October 28-31, 2000, Satellite Symposium October 29, 2000

3. *PPARγinduced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University, Breast Cancer Center of Excellence, Winston Salem, NC, November 16, 2000

4. *PPARγinduced apoptosis requires de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Keystone Symposium: PPARs a transcription odyssey, Keystone, CO, February 2-9, 2001

5. *Mechanisms of 15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis in breast cancer cells.* Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Nashville, TN October 14-17, 2001

6. *PPARγdoes not mediate apoptosis in breast cancer cells: role for lipid and protein modification.* South Eastern Regional Lipid Conference, Cashiers, NC, November 7-9, 2001

- Presentations
  1. *PPARγinduced biologic responses require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, Winston-Salem, NC, August 11-12, 2000

  2. *15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis in suppressed by a PPARγdominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

  3. *Mechanisms of Apoptosis in breast cancer cells: 15deoxy$\Delta^{12,14}PGJ_2$ and PPARγ.* University of Colorado Health Sciences Center, Denver, CO, February 9, 2001.

  4. *PPARγdoes not mediate apoptosis in breast cancer cells: role for lipid and protein modification.* South Eastern Regional Lipid Conference, Cashiers, NC, November 7-9, 2001

- Awards
  1. Comprehensive Cancer Center Award: Best graduate student presentation (monetary award) *PPARγ induced biologic changes require de novo gene expression that is suppressed by a dominant negative mutant in breast cancer cells.* Wake Forest University Cancer Center Faculty Retreat, August 11-12, 2000

  2. Avanti Founder's Award: Best graduate student presentation (monetary award and conference expenses) *15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis in suppressed by a PPARγdominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

  3. Avanti Founder's Award: Outstanding graduate student presentation (monetary award and conference expenses) *15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis in suppressed by a PPARγdominant negative.* South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

  4. Cayman Chemical Travel Award (monetary award) *Mechanisms of 15deoxy$\Delta^{12,14}PGJ_2$ induced apoptosis in breast cancer cells.* Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Nashville, TN October 14-17, 2001

  5. Avanti Founder's Award: Outstanding graduate student presentation (monetary award and conference expenses) *PPARγdoes not mediate apoptosis in breast cancer cells: role for lipid and protein modification.* South Eastern Regional Lipid Conference, Cashiers, NC, November 7-9, 2001

- Funding applied for based on work supported by this award
  1. Susan G. Komen Breast Cancer Foundation Dissertation Award. *PPARγInduced Apoptosis Requires de novo Gene Expression in Breast Cancer Cells: searching for key molecular targets.* (submitted March 15, 2001)

2. Wake Forest University Comprehensive Cancer Center. *PPARγand soy phytoestrogens as possible therapy for breast cancer.* $10,000 (submitted March 15, 2001)

3. Wake Forest University Comprehensive Cancer Center. *Adaptation of Cancer Cells to Oxidative Stress and the Anti-Cancer Mechanism of 15-deoxy-prostaglandin J_2.* $25,000 (submitted November 16, 2001, not funded)

Conclusions

Naturally occurring derivatives of arachidonic acid metabolism are potent and effective activators of PPARγ. The most potent of these derivatives is 15deoxy$\Delta^{12,14}PGJ_2$ ($15dPGJ_2$), the dehydration and isomerization product of prostaglandin D_2 (PGD_2). $15dPGJ_2$ induces PPARγ mediated transcriptional activation leading to the synthesis of critical gene products involved in cell cycle arrest and apoptosis. Of these gene products, expression of the cyclin dependent kinase inhibitors, p21 and p27, is associated with marked cell cycle arrest with subsequent apoptosis involving caspase-3. Although $15dPGJ_2$ inhibits NFκB mediated transcription. this likely represents a minor contribution to $15dPGJ_2$ induced apoptosis in breast cancer cells. Other candidate mechanisms include inhibition of the ubiquitin proteosome and generation of novel oxidized lipid intermediates. Investigations into altered fatty acid metabolism pathways are underway and may yield clues as to how arachidonic acid derivative exert such potent anti-neoplastic activity in breast cancer cells. $15dPGJ_2$ may represent a novel class of therapeutic molecules for the treatment of breast cancer.

References

1. **Clay CE,** Namen AM, Fonteh AN, Atsumi G, High KP, Chilton FH. (2000) 15deoxyΔ12,14PGJ$_2$ induces diverse biological responses via PPARγ activation in cancer cells. *Prostaglandins and Other Lipid Mediators* 62:23-32
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Clay CE, Namen AM, Atsumi G, Willingham MC, High KP, Kute TE, Trimboli AJ, Fonteh AN, Dawson PA, Chilton FH. (1999) Influence of J Series Prostaglandins on Apoptosis and Tumorigenesis of Breast Cancer Cells. *Carcinogenesis* 20:1905-1911 (published prior to DOD funding).

Appendices

Appendix 1: **Mechanisms of 15deoxy$\Delta^{12,14}PGJ_2$ induces apoptosis in breast cancer cells.** $15dPGJ_2$ induced apoptosis in breast cancer cells requires the expression of critical gene products, such as p21 and p27. However, $15dPGJ_2$ also induces the generation of reactive oxygen species which may act on free arachidonic acid (AA) to yield novel nuclear receptor agonists. Moreover, $15dPGJ_2$ inhibits key survival signaling protein, such as NFκB and AKT/PKB, and inhibits isopeptidase activity of the ubiquitin proteosome. Together these data show that the extraordinary biological activity of $15dPGJ_2$ is a result of PPARγ-dependent and independent mechanisms. Further research is warranted to discern the predominant mechanisms of $15dPGJ_2$-induced apoptosis in breast cancer cells.