Award Number:  DAMD17-00-1-0489

TITLE:  A Potential Therapeutic Role of J Series Prostaglandins in PPARy Mediated Treatment of Breast Cancer

PRINCIPAL INVESTIGATOR:  Arta Monir Monjazeb
Dr. Floyd H. Chilton
Dr. Kevin P. High

CONTRACTING ORGANIZATION:  Wake Forest University School of Medicine
Winston-Salem, NC  27157

REPORT DATE:  June 2003

TYPE OF REPORT:  Annual Summary

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release;
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A Potential Therapeutic Role of J Series Prostaglandins in PPARy Mediated Treatment of Breast Cancer

Naturally occurring derivatives of arachidonic acid metabolism are potent and effective activators of PPARy. The most potent of these derivatives is 15deoxyPGJ2 (15dPGJ2), the dehydration and isomerization product of prostaglandin D2 (PGD2). 15dPGJ2 induces PPARy 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. However, apoptosis induced by 15dPGJ2 is unlikely to be PPARy mediated as demonstrated by studies with dominant negative forms of this receptor. To further elucidate how AA derivatives such as 15dPGJ2 induce apoptosis in breast cancer cells investigations into AA metabolic pathways were undertaken. We demonstrate that intracellular accumulation of AA induces apoptosis in cancer cells by activating the AP-1 family of nuclear transcription factors. Given the anti-cancer efficacy of therapies which alter AA metabolism, such as NSAIDs, further investigation into 15dPGJ2 and other facets of the AA metabolic pathway are warranted.
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Appendix 1: Mechanisms of action of 15deoxy\(\Lambda^{12,14}\)PGJ\(_2\) in breast cancer cells
**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. 15deoxyΔ12,14PGJ₂, 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. PGE2, 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 D2 metabolism, 15deoxyΔ^12,14PGJ2 (15dPGJ2), 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). In addition, 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, β/δ, and γ) from GlaxoSmithKlein (GSK). These compounds are 10,000 fold more selective for their respective receptor than for other nuclear receptors. 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 (Figure 1).

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 15dPGJ2. Of these, the expression of the cyclin dependent kinase inhibitors p21\textsuperscript{Waf1/Cip1} (p21) and p27\textsuperscript{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 (Figure 2) and will establish cell lines that express a dominant negative form of p21. Additionally, Mr. Clay has followed up on published reports of the effects of 15dPGJ2 in other cell systems to devise a potential mechanism by which 15dPGJ2 or other cyclopentenone prostaglandins, may exert such potent anti-neoplastic activity in a variety of cancer cell types (Appendix 1). These studies have resulted in a manuscript which has been published in The Journal of Biological Chemistry (3). Mr. Clay will continue this line of investigation to include other gene products and further elucidate the mechanisms described. Furthermore, Mr. Clay has established breast cancer cell lines that express a dominant negative form of PPARγ. He has shown that transcriptional activation of PPARγ by 15dPGJ2 is blocked in these cells (Figure 3). More recently Mr. Monjazeb, in conjunction with Mr. Clay, has utilized these dominant negative forms of PPARγ as well as pharmacologic inhibitors of PPARγ to demonstrate that the apoptotic potential of 15dPGJ2 is independent of PPARγ activation in cancer cell lines. These results have been published in the Journal of Lipid Research. Furthermore, our results, as well as many recent published reports, suggest that the anti-neoplastic effects of 15dPGJ2 may be linked to AA metabolism in general as it has been demonstrated that other products of AA oxidation also have potent effects on cancer cell growth and viability. These results are outlined in a peer review published in Prostaglandins Leukot Essent Fatty Acid.

Aim 3: Given the findings described in Aim 2, we chose to expand Aim 3 to investigating the role of AA and its metabolites in neoplastic cells rather than focusing on the metabolism of 15dPGJ2 alone. We examined the anti-tumor effects of three distinct AA metabolic enzyme inhibitors Triacsin C, PLT 98625, and NS-398, which inhibit Fatty Acid Coenzyme-A Ligase 4 (FACL-4), Coenzyme-A Independent Transacylase (CoA-IT), and Cyclooxygenase (COX), respectively. We found that inhibition of AA metabolism had potent anti-neoplastic effects in a number of cancer lines including the MDA-MB-231 cell line and induced caspase-3 activation and apoptosis. We also found that these inhibitors potentely increase intracellular accumulation of AA and that apoptosis was intimately linked with this apoptosis. Our results suggest that inhibitors of AA metabolic enzymes induce cancer cell apoptosis by increasing levels of intracellular unesterified AA and indicate that combination treatment strategies utilizing these inhibitors may represent a novel approach to blocking cancer cell growth. These results are included in a manuscript under preparation for submission to the Journal of Lipid Research.

We performed further studies to examine downstream pathway(s) whereby high cellular burdens of unesterified AA promote apoptosis. We found that many previously hypothesized pathways, including the conversion of accumulated AA to cytotoxic products or AA induced ceramide generation, are unlikely to play a key role in the apoptosis induced by AA metabolic enzyme inhibitors; however, further study is required. Given the ability of AA and it's metabolites to augment gene transcription we hypothesized that AA accumulation resulting from AA metabolic enzyme inhibition induces apoptosis by regulating gene expression. One primary candidate was PPAR - mediated gene regulation because AA and its metabolites are PPAR ligands and the PPARs are known to be involved in cancer cell apoptosis. We found that AA metabolic inhibitors and exogenous AA do indeed augment PPAR - mediated gene transcription but that this phenomenon is unrelated to the apoptotic induction by these inhibitors. These results agree with our previous findings with regards to 15dPGJ2. Using Affymetrix gene array technology we were able to determine that intracellular accumulation of AA and its oxidation to various AA metabolites likely induce apoptosis in neoplastic cells by activating the AP-1 family of stress response transcription factors. These results are included in a manuscript in preparation for submission to Carcinogenesis.
Key Research Accomplishments

- 15deoxyΔ^{12,14}PGJ2 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 from increased proliferation to differentiation and apoptosis.
- 15deoxyΔ^{12,14}PGJ2 induced apoptosis requires de novo expression of critical gene products.
- Dominant negative expression of PPARγ completely abrogates transcriptional activation induced by 15deoxyΔ^{12,14}PGJ2.
- The mechanism of action of 15deoxyΔ^{12,14}PGJ2 is not limited to PPARγ activation. 15deoxyΔ^{12,14}PGJ2 can inhibit NFκB, activate PPARγ and can stimulate reactive oxygen species generation. Together, these events lead to induced expression of key gene products that are involved in PPARγ mediated apoptosis in breast cancer cells.
- 15deoxyΔ^{12,14}PGJ2 is metabolized to polar derivatives by breast cancer cells.
- 15deoxyΔ^{12,14}PGJ2 induced cancer cell apoptosis is independent of PPARγ.
- Inhibition of AA metabolism induces intracellular accumulation of AA in neoplastic cells. Apoptosis induced by inhibition of AA metabolic enzymes such as COX is likely due to the accumulation of intracellular AA.
- The AP-1 family of nuclear transcription factors are likely involved in neoplastic cell apoptotic signaling in response to AA and its metabolites.

Reportable Outcomes

- Manuscripts

- Abstracts
  1. *PPARγ induced 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. 15deoxyΔ^{12,14}PGJ2 inhibits breast cancer cell proliferation via PPARγ activation. *International Society for Preventive Oncology, 5th International Meeting*, Geneva, Switzerland, October 28-31, 2000, Satellite Symposium October 29, 2000
  3. *PPARγ induced 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. **PPARγ induced 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

- **Presentations**
  1. **PPARγ induced 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. **15deoxyΔ¹²,¹⁴PGJ₂ induced apoptosis in suppressed by a PPARγ dominant negative.** South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

- **Development of cell lines**
  1. PPARγ Dominant Negative
  2. IκBα Dominant Negative
  3. p21 Dominant Negative

- **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) **15deoxyΔ¹²,¹⁴PGJ₂ induced apoptosis in suppressed by a PPARγ dominant negative.** South Eastern Regional Lipid Conference, Cashiers, NC, November 1-3, 2000

- **Funding applied for based on work supported by this award**
  1. Susan G. Komen Breast Cancer Foundation Dissertation Award. **PPARγ Induced 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. **PPARγ and soy phytoestrogens as possible therapy for breast cancer.** $10,000 (submitted March 15, 2001)
Conclusions

Naturally occurring derivatives of arachidonic acid metabolism are potent and effective activators of PPARγ. The most potent of these derivatives is 15deoxyΔ12,14-PGJ₂ (15dPGJ₂), the dehydration and isomerization product of prostaglandin D₂ (PGD₂). 15dPGJ₂ 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. However, apoptosis induced by 15dPGJ₂ is unlikely to be PPARγ mediated as demonstrated by studies with dominant negative forms of this receptor. To further elucidate how AA derivatives such as 15dPGJ₂ induce apoptosis in breast cancer cells investigations into AA metabolic pathways were undertaken. We demonstrate that intracellular accumulation of AA induce apoptosis in cancer cells by activating the AP-1 family of nuclear transcription factors. Given the anti-cancer efficacy of therapies which alter AA metabolism, such as NSAIDs, further investigation into 15dPGJ₂ and other facets of the AA metabolic pathway are warranted.
References


Appendices
Appendix 1: Mechanisms of 15deoxyΔ^{12,14}PGJ\textsubscript{2} induces apoptosis in breast cancer cells. 15dPGJ\textsubscript{2} induced apoptosis in breast cancer cells requires the expression of critical gene products, such as p21 and p27. However, 15dPGJ\textsubscript{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\textsubscript{2} inhibits key survival signaling protein, such as NFκB and AKT/PKB, and inhibits isopeptidase activity of the ubiquitin proteasome. Together these data show that the extraordinary biological activity of 15dPGJ\textsubscript{2} is a result of PPARγ-dependent and independent mechanisms. Further research is warranted to discern the predominant mechanisms of 15dPGJ\textsubscript{2}-induced apoptosis in breast cancer cells.

15deoxyΔ^{12,14}PGJ\textsubscript{2} or other cyclopentenone prostaglandins

PLA\textsubscript{2}
Free AA
NSAIDs
Triacsin C
CoA-IT

Oxidized Lipids

NFκB, PPARγ

NFκB, PPARγ

IκBα, IκBβ

IκBα, IκBβ

Proteolytic degradation

IAPs, COX-2
Anti-Apoptotic Genes

SOD, GSH, CAT, HO-1
Anti-Oxidative Genes

p21, p27
Pro-Apoptotic Genes

Caspase-3

Apoptosis