

Identification and Functional Activation of Peroxisome Proliferator-Activated Receptor
in Human Upper Aerodigestive Cancer

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Abstract

Squamous cell carcinoma of the head and neck (SCCHN) is an aggressive malignancy whose carcinogenesis occurs in multiple stages years to decades after carcinogen exposure. In spite of continued advances in the understanding of molecular biology of SCCHN and the introduction of a multitude of multi-modality treatment protocols, the 20-50% survival of stage III and IV disease has not changed appreciably in over twenty years. Efforts to treat or prevent recurrence have predominantly involved the use of cytotoxic chemotherapy, however the use of retinoids as a chemoprevention agent has been clinically assessed. Success has been limited by toxicity of retinoids and reversal of differentiation changes upon cessation of treatment. Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors belonging to the steroid/thyroid/retinoic acid nuclear receptor superfamily. PPARs primarily target genes involved in lipid homeostasis; one isoform, PPAR γ , directs the differentiation of precursor cells into adipocytes. PPAR γ heterodimerizes with RXR α to form a functional transcription factor. The recognition of ETYA as a PPAR activator along with the previously-discovered anti-cancer and differentiation effects of this agent invoked the possibility that a differentiation strategy could be used in the treatment in SCCHN. This study examined PPAR γ in SCCHN. PPAR γ protein was expressed in SCCHN cell lines. Treatment of two cell lines with three chemically distinct ligands of PPAR γ caused dose-dependent inhibition of proliferation. Cells treated with these agents caused cytoplasmic lipid vacuole accumulation consistent with adipogenic phenotype shift. Electromobility supershift analysis demonstrated DR-1 consensus sequence binding activity in SCCHN nuclear extracts. The more-specific cyp4a1 oligonucleotide also demonstrated binding, the amount of which was upregulated with treatment. Supershift analysis demonstrated presence of PPAR γ in nuclear extracts. Functional activation was assessed using dual transfection reporter gene assays, which demonstrated dose dependent increased PPRE activation with treatment using each agent. We conclude that PPAR γ ligands may represent a class of drugs which have value in the treatment of SCCHN.

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1.0. INTRODUCTION

In spite of continued advances in the understanding of molecular biology of squamous cell carcinoma of the head and neck (SCCHN) and the introduction of a numerous multi-modality treatment protocols, the 20-50% survival of stage III and IV disease has not changed appreciably in over twenty years [1]. Promising biologic targets for improving treatment have included nuclear receptor targeting with retinoids to prevent primary and secondary tumors. Success has been limited by toxicity of retinoids and reversal of differentiation changes upon cessation of treatment [2, 3]. Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the steroid/thyroid/retinoic acid nuclear receptor superfamily that are a second promising target in this family. One isoform, PPAR γ , heterodimerizes with RXR α to form a functional transcription factor, and directs the differentiation of precursor cells into adipocytes [4].

Several natural and synthetic fatty acids, including the arachidonic acid analog eicosatetraenoic acid (ETYA), are PPAR γ activators [5]. Early experiments in several tumor types demonstrated the ability of ETYA and related agents to dramatically downregulate cell growth and promote features consistent with differentiation in a variety of cell lines [6-11]. ETYA is a pleiotropic compound that also is a lipoxygenase inhibitor and previous experiments demonstrated that structurally distinct lipoxygenase inhibitors demonstrated a variety of antineoplastic features not entirely explained by the inhibition of lipoxygenase alone [12-15].

As a PPAR γ activator, it is likely that several anticancer effects of these drugs may be attributable to PPAR γ activation [16]. PPAR γ was identified as a nuclear transcription factor in adipocytes in 1994 [17, 18] and strongly directs adipocyte differentiation, even when ectopically expressed in fibroblasts [18, 19]. While mostly explored in the context of adipocyte differentiation, lipid metabolism, and inflammation, PPAR γ has been shown to have an important role in carcinogenesis. Cell line studies have documented an anti-proliferative role of PPAR γ agonists through cell-cycle arrest through upregulation of

CDK inhibitors in epithelial-derived human cancer cell lines including prostate [20], breast [21], colon [22], and lung [23]. Inhibition of growth has been linked to G1 phase cell cycle arrest through suppression of cyclin D1 expression [24-26]. The potential role for PPAR to modulate the early events in carcinogenesis has spawned considerable interest in this transcription factor as a target for chemoprevention [27].

Cancer largely represents a lack of differentiation; therefore, the possibility of driving premalignant or cancerous cells into a terminally differentiated state is an attractive potential therapeutic strategy. Differentiation therapy has been successfully used in the treatment of acute promyelocytic leukemia exhibiting the t[15, 28, 29] translocation with all-*trans*-retinoic acid [30, 31], hairy cell leukemia, and childhood neuroblastoma [32, 33]. Receptor agonism with retinoids has shown mixed results in clinical therapies for solid tumors or chemoprevention [2, 34, 35]. Because previously unrecognized PPAR γ ligands have been shown to induce morphologic changes associated with differentiation in earlier experiments in our lab, as well as anti-cancer effects in other cancer models, we examined SCCHN cell lines and tumor specimens for the expression of PPAR γ . Treatment of two cell lines with three chemically distinct ligands of PPAR γ caused dose-dependent inhibition of proliferation and cytoplasmic lipid vacuole accumulation and morphologic changes consistent with cellular differentiation with an adipogenic phenotype shift. Electromobility supershift analysis demonstrated specific DR-1 and cyp4A1 consensus sequence binding activity in nuclear extracts that was upregulated with PPAR γ ligand treatment. PPAR γ and RXR α were identified as the predominant binding partners in control and ligand-stimulated nuclear extracts. Functional activation of the PPAR response element (PPRE) demonstrated increased PPRE activation with each ligand in a 3x PPRE reporter and the fat specific protein aP2. Further experiments demonstrated PPAR family members were decreased but present in a population of human aerodigestive specimens compared to normal oral mucosa. We conclude that PPAR γ ligands represent a class of drugs that have value in the treatment of SCCHN and its precursor lesions.

2.0. LITERATURE REVIEW

2.1. Peroxisomes

Peroxisomes are subcellular organelles present in virtually all eukaryotic cells ranging from eukaryotic microorganisms to plants and animals. Peroxisomes perform a diverse metabolic functions including the synthesis of plasmalogens, bile acids, cholesterol and dicholol, and the oxidation of fatty acids, including very long chain fatty acids and branched chain fatty acids. Peroxisomes are also responsible for the metabolism of purines, polyamines, amino acids, glyoxylate and reactive oxygen species [36].

Peroxisome proliferators are a diverse group agents – both industrial and pharmaceutical – originally identified as in vitro exposure-related inducers of size and number of peroxisomes in rat hepatocytes. This correlates with transcriptional activation of fatty acid metabolizing enzymes. Sustained exposure to some peroxisome proliferators causes an increased incidence of liver tumors in mice and rats [37]. Evidence implicates a subset of nuclear receptor in mediating some or all of the changes associated with peroxisome proliferator exposure. These receptors respond to activation by modulating gene transcription of lipid metabolism genes and peroxisome proliferation.

2.2. Peroxisome Proliferator-Activated Receptors (PPARs)

Maintenance of caloric homeostasis is a basic function of all life. Living organisms must cope with unpredictable access to energy sources and variable energy needs. Immediate metabolic changes are managed within the context of existing enzymatic systems within the organism; longer-term energy management, however, depends on gene transcription. The modulation of gene transcription in response to the metabolic environment requires transducer agents which represent the parameters of metabolic needs of the organism, such as fatty acid or glucose concentrations. The link between these parameters and gene transcription are the PPARs.

A major advance in the discovery of PPARs came from the analysis of tissue-specific enhancer for the adipocyte P2 (aP2) gene [38]. The main transcriptional regulator of both

the aP2 and PEPCK genes was identified as ARF6, whose binding sequence was similar to the DR-1 hormone response element of a member of the nuclear hormone receptor family [39].

Peroxisome proliferator-activated receptors are nuclear hormone transcription factors assigned to the steroid/thyroid/retinoid superfamily based on their highly conserved DNA- and ligand-binding domains. In 1990, a member of the nuclear receptor superfamily was cloned from mouse liver [40]. As with other members of the vertebrate steroid nuclear hormone receptor superfamily, the PPAR subfamily comprises distinct isoforms: PPAR α , PPAR β/δ , and PPAR γ . Human genes for the alpha and gamma subtypes have been mapped to chromosome regions 22q12-q13.1 and 3p25 respectively. Nuclear receptors characteristically exhibit multiple functional domains; PPARs exhibit four functional domains – A/B, C, D, and E/F. The A/B region is poorly conserved and encodes a ligand-independent transcriptional domain. The C domain is the DNA binding domain (DBD) and is the most-conserved portion of the receptor. It contains two zinc finger DNA binding motifs which target the receptor to DNA sequences, termed peroxisome proliferator response elements (PPREs) in a highly specific manner. The specificity of binding is determined by the P-box amino acids located at the carboxy end of the first zinc finger. The D domain serves as a hinge connecting the C and E domains.

The E domain comprises the ligand binding domain (LBD). This domain imparts the transduction function to the transcription factor by converting hormone signal to transcriptional activation. This structure consists of 12 alpha helices and undergoes conformational change upon ligand binding, swinging closed over the ligand in a “mouse trap” fashion. Additionally, this domain is involved in dimerization, nuclear localization, and association with modulators of transcription [41]. The ligand-induced conformational change, which includes the AF-2 helix across the opening of the binding pocket, allows the appropriate orientation for binding of a co-activator protein termed the steroid receptor coactivator (SRC-1). At $\sim 1300\text{\AA}^3$, the PPAR ligand binding pocket is 2-3 times larger than those of other nuclear hormone receptors; this may explain the ability

of these receptors to accommodate the structurally diverse array of chemical activators [42].

2.3. DNA Binding Properties of PPARs

Nuclear hormone receptors bind to DNA by recognizing target sequences composed of six nucleotides, called core recognition motifs. The six-nucleotide target sequence for PPARs is identical to that of members of the TR/RAR class: AGGTCA. Members of these classes bind to DNA as dimers with both partners contacting DNA; thus, two copies of the target sequence are necessary for binding and gene activation. A functional pair of core recognition motifs is termed a hormone response element (HRE) or direct repeat sequences (DR). The orientation and spacing of the motifs determine which nuclear hormone receptors bind the HRE [43]. Within the heterodimer, the PPAR component binds to the extended 5' half-site of the PPRE, while RXR binds to the 3' hexamer[44].

PPARs obligately heterodimerize with RXR; they do not function as monomers or homodimers. Thorough in vitro binding studies examining the spacing between direct repeats demonstrated that a single base pair is the optimal pattern (AGGTCA-n-AGGTCA). This binding preference confers specificity of PPREs (DR-1) from other HRE such as those of VDR (DR-3), TR (DR-4), and RAR (DR-5). The 5' flanking region of endogenous PPREs are also critical to the binding of the heterodimer. Also, the PPAR:RXR heterodimer demonstrate a strong preference for an A:T base pair DR-1 spacer. Interestingly, co-transfection experiments have demonstrated PPAR:RXR heterodimer response to both PPAR activators and 9-cis-retinoic acid, implying that whatever allosteric changes occurring with PPAR activation do not obviate the binding of the retinoid ligand binding region.

2.4. PPAR Isoforms and Tissue Distribution

Three distinct PPAR isoforms have been identified, each of which has a distinct tissue distribution and physiologic function related to lipid homeostasis. PPAR α is highly expressed in liver, kidney, intestine, and is a regulator of fatty acid metabolism. It may

also play a role in modulating inflammatory response [45]. It is also highly expressed in brown fat. PPAR β/δ has a diffuse tissue distribution; its physiologic role has not been clearly elucidated. PPAR γ has two isoforms generated by differential splicing: PPAR γ 1 and PPAR γ 2. Each is generated from the same gene through alternative promoter usage and splicing. PPAR γ 1 is found in liver, intestine, spleen, and adipose tissue; PPAR γ 2, which has 30 extra N-terminal amino acids, is found exclusively in adipose tissue.

2.5. PPAR γ and Adipose Differentiation

The terminal differentiation process of adipocytes from precursor cells involves a multistage process including an alteration of expression of several adipocyte-related proteins. This change depends upon the modulation of transcription of these proteins at the gene level. The discovery of a tissue-specific regulatory protein acting to modulate adipocyte cell development was a major advance in the study of adipogenesis. Two main protein families have been shown to play key roles in the differentiation of the adipocyte: PPARs and the CCAAT/enhancer-binding protein (C/EBP) family. Pre-adipocytes contain only trace amounts of PPAR γ and C/EBP proteins [29].

PPAR γ has been identified as a major controller of adipocyte differentiation. PPAR γ 2 expression follows the differentiation of precursor cells, beginning at low levels in pre-adipocytes, then markedly increases early in adipocyte differentiation [17]. Remarkably, transient transfection experiments in fibroblasts in which ectopic expression and activation of PPAR γ caused adipocyte conversion [18]. Target PPREs have been identified in the promoter regions of multiple enzymes involved in fatty acid transport and metabolism, including aP2 (mouse), fatty acid binding protein (FABP) (rat), apolipoprotein C-III (human), acy-CoA oxidase (rat), malic enzyme, phosphoenolpyruvate carboxykinase (rat), and others [46]. PPAR γ has strong binding affinity for most of these PPREs. Interestingly, PPAR α has weak binding affinity for the aP2 promoter, which is a late PPAR target gene containing a fat-specific enhancer bearing the response element ARE7, thereby possibly explaining the ability of PPAR α to generate adipocyte differentiation.

Definitive evidence of the critical role of PPAR γ in adipogenesis comes from experiments using PPAR γ knockout (KO) mice. Studies performed by Kubota et al. (1999) [47] and Miles et al (2000) [48]. PPAR γ $-/-$ mice were found to be completely absent of adipose tissue. PPAR γ $+/-$ mice were found to have a decreased overall mass of adipose tissue. Rosen et al. (1999) [49] demonstrated that injection of PPAR γ $-/-$ embryonic mouse cells into wild-type blastocysts produced chimeric mice composed exclusively of PPAR γ $+/+$ cells, thus indicating the requirement of PPAR γ expression for the development of this tissue.

Genetic studies in humans also add to the evidence that PPAR γ is a central controller of fat differentiation. A Pro115 Gln mutation in the NH₂-terminal ligand-independent activation domain of PPAR γ was identified in four morbidly obese patients. This mutation results in the inhibition of binding at Ser112 resulting in a permanently active PPAR γ . A more common Pro12 Ala substitution in the PPAR γ 2-specific exon B, resulting in a less active PPAR γ form, is associated with a lower body mass index [50-53]. This information provides significant evidence for the *in vivo* control of PPAR γ in adipogenesis.

The CCAAT/enhancer binding protein family is a group of leucine zipper proteins. C/EBP β and δ are expressed early and transiently in the process of adipocyte differentiation; expression then decreases in the terminal phase of differentiation, at which point C/EBP α is induced. Ectopic expression of C/EBP β in adipocytes caused hormone-independent adipogenic conversion, and in fibroblasts caused adipogenic conversion with hormonal stimulation [54]. Two C/EBP binding sites have been found in the PPAR γ 2 promoter. It is thought that adipocyte differentiation begins with hormonal induction C/EBP β and C/EBP δ , resulting in expression of PPAR γ . The presence of a ligand for PPAR γ permits the transcription of the multitude of adipocyte genes, including C/EBP α . It is possible that the C/EBP binding sites on the PPAR γ provide a positive feedback loop which maintains the adipocyte-differentiated state. Overexpression of C/EBP α alone can induce adipocyte differentiation in fibroblasts and antisense C/EBP α

inhibits terminal differentiation of adipocytes (reviewed in Brun, et al.). Furthermore, co-expression of C/EBP α and PPAR γ in fibroblasts causes high levels of differentiation without the need for exogenous activators. The serial expression of C/EBP β/δ , PPAR γ , C/EBP α

2.6. Regulation of PPAR γ

2.6.1. ADD1/SREBP. Other proteins are involved in the regulation of adipogenesis. One such protein is the sterol regulatory element binding protein (SREBP) 1, which binds the sterol regulatory element (SRE) 1 whose sequence is ATCACCCCAC. The transcription factor binds the SRE-1 as a homodimer; the SRE has been localized in the promoters of some genes involved in the regulation of cholesterol. Evidence of an adipocyte differentiation role for the rat homologue of SREBP, ADD1, is that ectopic expression of a dominant negative form inhibits adipocyte gene expression in 3T3-L1 preadipocytes [55]. Ectopic expression of ADD1/SREBP1 activates PPAR γ , possibly through generation of endogenous ligands.

2.6.2. 5' Flanking Region. The 5'-flanking region of the PPRE is important in the binding of the heterodimer elements to the PPRE. This was demonstrated by ordering 16 major natural PPRE's from strong to weak binding and examining the core DR-1 sequence and 5'-flanking regions. While the core motifs had a relatively uniform degree of conservation, the 5'-flanking region demonstrated a correlation between binding activity and number identities compared to the consensus sequence [46]. Furthermore, the PPAR γ -specific element ARE6 was able to bind PPAR α only if its 5'-flanking region was exchanged with that of the less selective HMG PPRE.

2.6.3. Protein Phosphatases. Protein phosphatases play a role in the modulation of nuclear transcription factor, including PPAR γ . A number of cellular proteins such as the positively acting cyclins and cyclin-dependent kinases (CDKs), or the negatively acting cyclin-dependent kinase inhibitors (CDKIs) govern cell cycle progression by controlling the activity of the retinoblastoma (RB) protein through phosphorylation. Consistent with

its role as a tumor suppressor, virtually all human cancers are associated with alterations in the RB pathway, either through inactivation of RB itself or the CDKI p16^{INK4a}, or through overexpression of cyclin D1 and CDK4 oncoproteins. In this pathway, the E2F transcription factor is a key downstream target of RB. Hypophosphorylated RB binds E2F and thereby down-regulates E2F activity, suggesting a model in which RB restricts cell cycle progression by restraining E2F. In fact, the interaction of RB with E2F correlates with the capacity of RB to arrest cell growth in the G1 phase . On the other hand, loss of RB-mediated control of E2F activity leads to progression into DNA synthesis [56].

The role of protein phosphatases in SCCHN relative to PPAR γ is of great interest. It has been demonstrated that selective loss of E2F/DP DNA binding and E2F/DP transcription factor activity with treatment of PPAR γ activator occurs. This was further investigated through mixed extract experiments in which extracts from untreated cells were mixed into extracts from PPAR γ activator (pioglitazone) treated cells, with drastic restoration of E2F/DP binding activity. However, when the PP inhibitor Okadaic Acid (OA) (3nM) was added, the restoration did not occur. The concentration suggests inhibition of PP2A only. To definitely distinguish between PP2A and PP1A, IP-2 was added; it inhibits PP1A at nanomolar concentrations with no effect on PP2A. In this case, IP-2 did not change EF-2 binding at 1 microgram concentrations. To further support the hypothesis that PP2A was inhibited in pioglitazone-treated cells, purified PP2A subunit was added to extracts of untreated and treated cells, with restoration of EF-2/DP binding in the latter only [57].

Precipitation studies were next performed which demonstrated dramatic increase in DP-1 phosphorylation with pioglitazone treatment. Further, activation of PPAR γ causes a dramatic decrease in PP2Ac, which corresponds with the decrease in EF2/DP binding activity. The physiological role of PPA2 as a cell cycle regulator is consistent with the hypothesis that PPAR γ -directed downregulation of PPA2 accounts for the cell cycle arrest and exit from cell cycle observed with pioglitazone treatment.

2.6.4. Protein Phosphatases and Gene Regulation. Evidence suggests that the phosphorylation state of PPAR γ itself affects its DNA binding capacity. The importance of protein tyrosine phosphatases is illustrated in the context of nuclear hormone receptor regulation, including PPAR γ . MAP kinase has been shown to be responsible for phosphorylation of PPAR γ , which inhibits its function. Activators of protein kinase A (PKA) can enhance PPAR activity in transfection experiments, and PKA has been shown to stabilize binding of liganded PPAR to DNA [30].

It has been recently shown that phosphorylation and activation of a powerful transcriptional co-activator of PPAR γ , PGC-1 (PPAR γ coactivator 1), is mediated by p38 MAPK. Several growth factors known to inhibit adipocyte differentiation were noted to cause mitogen-activated protein (MAP)-kinase-mediated phosphorylation of PPAR γ and resultant reduction of its transcriptional activity. Expression of a mutant transcription factor with a serine substitution at site 112 making it non-phosphorylatable increased the sensitivity of the cells to ligand-induced adipogenesis and resistance to mitogen induced inhibition of differentiation. The authors conclude that covalent modification of PPAR γ by serum and growth factors is as major regulator of the balance between cell growth and differentiation in the adipose cell lineage [19].

2.6.5. Activators. Heterodimers of PPAR γ and RXR α bind to a direct repeat hormone response element (DR-1) and direct expression of a variety of fat-specific target genes responsible for adipocyte differentiation. PPAR γ is specifically activated by a variety agents, including the thiazolidinedione class of antidiabetic drugs, certain fatty acids, prostaglandin J2 metabolites, especially 15-deoxy delta 12,14-PGJ2; indomethacin and other NSAIDS [5, 28, 58-60]. PPAR γ is the most selective PPAR, binding primarily to unsaturated fatty acids. The finding that certain endogenous prostaglandins, leukotriene and eicosanoid metabolites can activate PPAR γ suggest that PPAR γ has the capacity to participate a vast array of cellular events which are affected by these messenger molecules, including carcinogenesis.

3.0. DETAILED REVIEW

3.1. Epidemiology

An estimated 644,000 new cases of head and neck cancers are diagnosed each year worldwide; two thirds of these cases occur in developing countries. In the United States, malignancies of the head and neck account for 3.2% of all new cancers (39,750) and 2.2% of all cancer deaths (12,460) [61]. Men have a three-fold higher incidence compared to women and African-American suffer from this disease more commonly than whites. The five-year survival is better for whites than for African-Americans (61-64% vs 40-52%) [62].

Squamous cell carcinoma makes up more than 90% of the histology of cancer of the head and neck; most originate in the lip/oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx. The incidence of cancer at the base of tongue and the tonsils has increased, particularly for patients younger than 45 years of age; this has been a significant trend over the past ten years [63]. This changes has been ascribed to increasing prevalence of human papilloma-virus (HPV) infection in developing countries, the practice of oral sex, and the increasing multiplicity of sexual partners [63]. Compared with HPV-negative squamous cell carcinomas (SCCs), these patients have better overall survival (OS) and higher cure rates[64]

3.1.1. Risk Factors. Tobacco smoking and alcohol consumption are by far the most common risk factors for SCC of the head and neck. A 5-25 fold higher risk of developing head and neck cancers has been noted for heavy smokers compared to non-smokers. Alcohol further increases risk such that the risk of developing cancer for a patient with a 40-pack year tobacco use history in addition to 5 drinks/day alcohol use history, the overall risk for HNSCC is increased by 40 fold [65]. Nicotine and polycyclic aromatic hydrocarbons in tobacco are considered directly carcinogenic. [5] Mutation of the tumor suppression gene p53 tumor protein (TP53) has been associated with alcohol

and tobacco exposure [66]. Betel quid chewing, a common practice in parts of Asia, has been associated with oral leukoplakia and oral submucous fibrosis [66].

Dietary factors are also believed to be important risk factors for oral and pharyngeal cancers. Many epidemiological studies have demonstrated an association between HNSCC and vitamin A deficiency and iron deficiency of the Plummer-Vinson syndrome [67, 68].

Epstein-Barr Virus (EBV) and HPV have both been linked with HNSCC. EBV is associated with nasopharyngeal carcinoma (NPC), particularly with World Health Organization (WHO) type II nonkeratinizing and type III undifferentiated classifications; these are most prevalent in endemic areas of northern Africa and Asia [69, 70]. Human papillomavirus is recognized to play a role in the oncogenesis of HNSCC [71]. Both epidemiologic and molecular research has demonstrated that roughly 25% of all HNSCCs are associated with HPV; approximately 60% of oropharyngeal carcinomas – most notably tonsillar carcinoma – are HPV positive [72]. High risk HPV types have been identified as HPV 16, 18, and 31; these are known to be tumorigenic in human epithelial tissues. High risk HPV viral oncoproteins E6 and E7 promote tumor progression by inactivating TP53 and retinoblastoma tumor suppressor gene products, respectively [72]. These malignancies seem to be clinically and molecularly distinct from HPV-negative HNSCC. Human papilloma-virus positive are more likely to originate in the oropharynx, be poorly differentiated, exhibit basaloid features, and present at a lower T-stage than HPV-negative HNSCC [71-75].

Occupational exposure to chromium, nickel, radium, mustard gas, and byproducts of woodworking and leather tanning has also been associated with cancers of the sino-nasal regions [76, 77].

Table 1. Head and Neck Cancer Risk Factors

| <u>Table 1. Head and Neck Cancer Risk Factors</u> | |
|---|---|
| Substance use: | |
| | Tobacco (primary risk factor) |
| | Smoking |
| | Chewing tobacco |
| | Secondhand smoke |
| | Ethanol |
| | Ethanol + tobacco (synergistic effect) |
| Dietary | |
| | Vitamin A deficiency |
| | Iron deficiency associated with Plummer-Vinson syndrome |
| Viruses | |
| | Human papillomavirus (HPV) types 16,18,31. |
| | Epstein-Barr virus (EBV) |
| Occupational exposure | |
| | Asbestos |
| | Nickel |
| | Chromium |
| | Radium |
| | Mustard gas |
| | Byproducts of leather tanning and woodworking |

3.1.2. Tumorigenesis. Tumorigenesis is related to specific genetic alterations of the upper respiratory tract caused by sustained exposure to carcinogenic material. Over the past ten years, the molecular understanding of HNSCC has been a major focus of investigation. The following are important areas of discovery with therapeutic implications highlighted.

Cyclin D1 (CCND1) is an oncogene that activates cell-cycle progression; this is amplified in 30-50% of HNSCC tumors [78]. Amplification and overexpression of CCND1 have been linked with early recurrence, more advanced disease, and shortened survival [78].

The TP53 tumor suppressor gene is mutated in 40-60% of patients with HNSCC; this mutation is associated with the progression from premalignancy to invasive disease [79]. Tumors with TP53 mutations have been shown to be more likely to recur early. One study demonstrated that in patients with TP53 mutations in the primary tumor that were subsequently detected in the surgical margins had early recurrences compared with patients with all tumor margins free of this mutation [80, 81]. Compared to patients who abstained from tobacco and alcohol, TP53 mutations were more common in patients using these substances. It is noteworthy that patients with HPV-positive tumors were less likely to have TP53 mutations. Poeta et al., [82] studied 560 patients who underwent primary resection of their HNSCCs; he observed a significant association (independent of stage) between TP53 mutational status and survival [82].

The gene CDKN2A (previously known p16), which is an inhibitor of cyclin-dependent kinase, is involved in regulating the cell cycle. Loss of chromosomal region 9p21 results in inactivation of the CDKN2A gene; this loss of function is the most common genetic change that occurs early in the progression of HNSCC malignancies [83].

Epidermal growth factor receptor (EGFR) expression was recently discovered in HNSCC tumors; EGFR is highly expressed in upward of 95% of HNSCCs. Increased expression of EGFR protein and its receptor, transforming growth factor alpha, is associated with poor prognosis [84]. Epidermal growth factor receptor has been linked to increased resistance to radiation, but no correlation exists to treatment response with new targeted agents, such as cetuximab and ertolinib. Increased in situ-detected EGFR gene copy numbers represents a negative prognostic factor and are associated with decreased progression-free survival (PFS) and decreased OS [85]. The variant III EGFR truncation mutation which consists of an in-frame deletion of exons 2-7 from the extracellular domain has recently been shown to predict resistance to EGFR inhibitors such as cetuximab [86].

3.2. Anatomy and Clinical Presentation

The most common location of HNSCC includes the oral cavity, oropharynx, nasopharynx, hypopharynx, and larynx [87]. The oral cavity is defined as the buccal mucosa, upper and lower alveolar ridge, retromolar trigone, floor of mouth, hard palate, and anterior two thirds of the tongue. The oropharynx is defined as the base of the tongue, tonsils, soft palate, uvula, posterior pharyngeal wall and lateral pharyngeal walls. The nasopharynx is defined as the space behind the nasal cavity and has a vault, posterior wall and lateral walls that include the fossa of Rosenmuller and mucosa covering the torus tubarius forming the orifice of the eustachian tube. The hypopharynx is located between the oropharynx and the cervical esophagus. It is divided into three parts: the pyriform sinus, the lateral and posterior walls, and the post-cricoid areas. The larynx is divided into three regions, including the supraglottic, glottic, and subglottic larynx.

Early stage symptoms are typically vague and minimal physical findings are evident at diagnosis; clinical presentation depends on the primary site involved [88]. Oral cavity cancer patients typically present with nonhealing sores or ulcers and pain; oropharynx involvement may result in symptoms of sore throat, chronic dysphagia, persistent odynophagia, or otalgia. Patients with cancer of the hypopharynx usually present with more advanced disease with symptoms of dysphagia, otalgia, hoarseness, and frequently cervical lymphadenopathy. Among patients with laryngeal cancer, the subsite involved will influence presentation. In most cases, these cancers present at early stages and are frequently cured. Symptoms at presentation include persistent hoarseness and later shortness of breath. Supraglottic tumors normally have more advanced disease and neck involvement [88].

Nasal cavity and paranasal sinus cancers are associated with symptoms of sinusitis, unilateral nasal airway obstruction, and epistaxis. Typical symptoms of NPC include recalcitrant otitis media, nasal obstruction, epistaxis, and cranial nerve palsies [88].

Physical examination of the head and neck should include visualization of all mucosal surfaces with attention to ulcers, submucosal masses, and surface irregularities.

Bimanual palpation of the floor of mouth and palpation of the neck should be performed. Careful examination of lymph nodes in regions I-V should be undertaken. Lymph node level I includes the submental and submandibular lymph nodes. Level II lymph nodes include the upper jugular lymph nodes. Level III includes the mid-jugular nodes; Level IV includes the lower jugular nodes. Level V includes the posterior triangle lymph nodes.

In the clinical scenario of a patient presenting with a painless cervical mass, the location of the enlarged lymph node may indicate the primary site of the cancer. Oral cavity cancers usually spread to level I. NPC frequently spread to level II and V. Laryngeal cancers often spread to levels II and III. For a variety of reasons, primary glottic and laryngeal cancers rarely spread to the neck nodes. Neck involvement is meaningful in predicting prognosis, as it generally reduces cure rates by 50% for a given T-stage.

3.3. Diagnosis and Treatment

Irregularities of mucosa found on physical examination and malignant lymph nodes with unknown primary site require further investigation. This should prompt complete endoscopy (flexible nasopharyngolaryngoscopy, esophagoscopy, and possibly bronchoscopy) as well as biopsies of any detected abnormality. In the setting of cancer of unknown primary site, directed biopsies of the nasopharynx, hypopharynx, tongue base, and possible tonsillectomy should be performed [89, 90]. Having established a diagnosis, the extent and location of disease must be determined to allow for precise staging. Next, imaging should be employed to assess for the possibility of distant metastasis. Initial evaluation can include high-resolution computed tomography (CT) or magnetic resonance imaging (MR); contrast provides higher yield in both cases. Imaging of the chest is recommended for patient with advanced neck disease, as they are at increased risk for distant metastases; high-risk tobacco users should also undergo chest imaging to screen for second primary tumors [91, 92].

Positron emission tomography (PET) has emerged as an important method in staging of HNSCC. This modality is Medicare-approved both for initial staging and restaging after treatment [93-97]. In patients presenting with unknown primary tumors of the head and neck, it can be used to localize the primary tumor; it can also be used to detect persistent disease after treatment. It is often helpful in assessing equivocal findings on routine imaging studies [94] Sensitivity and specificity of PET scanning for the detection of nodal metastases of 90% and 94% respectively, versus 82-85% for high-resolution CT.

3.3.1. Staging. Upon completion of comprehensive initial evaluation, staging is assessed using the American Joint Commission on Cancer (AJCC) TMN staging system [98]. Head and neck cancer T-staging differs based on primary cancer site; the N staging is common for all subsites with the exception of nasopharynx. The M staging is common to all sites. Stage IV is partitioned into stage IVA (advanced resectable disease), Stage IVB (advanced unresectable disease), and Stage IVC (advanced metastatic disease).

3.3.2. Treatment Options. Between 1960 and 1980, surgery and radiation therapy (RT) were considered the only effective treatment for primary curative management of HNSCC. A major paradigm shift in the treatment of HNSCC began in 1991 with the presentation of the Department of Veterans Affairs Laryngeal Cancer Study [99], which introduced the concept of organ preservation by comparing induction chemotherapy followed by RT to surgery followed by RT in patients with respectable stage III or IV laryngeal cancer. Researchers observed no significant difference between these groups after more than 10 years of follow up from original publication as well as preservation of the larynx in approximately two-thirds of survivors. In the pursuit of organ preservation, studies combining chemotherapy and RT were conducted with this paradigm of treatment modality sequencing optimization being the major focus of study [100, 101]. In 2003, the results from the RTOG 91-11 trial were published. RTOG 91-11 involved three arms which randomized patients with locally advanced respectable laryngeal cancer to 1) RT alone vs 2) induction chemotherapy followed by RT vs 3) concurrent chemoradiation.

Locoregional control and laryngeal preservation were significantly better in the concurrent chemoradiation arm than in the other 2 arms. Overall survival in these groups were statistically similar with a rate of 55%. Based on these results and those of other studies, concurrent chemoradiation became the standard of care for patients with locally advanced laryngeal carcinoma, with a goal of organ preservation. This treatment paradigm was used for all locally advanced subsites within the head and neck [102].

More recent advances in the management of HNSCC have included targeted therapy and concurrent RT. A 2006 trial reported results of a randomized trial exploring the use of definitive RT and concurrent cetuximab versus RT alone [103] A significant improvement in PFS and OS was observed with the addition of cetuximab. This combination is being applied in patients who are not candidates for therapy with platinum and radiation because of medical comorbidities.

The treatment of HNSC is centered on a multidisciplinary approach was then experienced team including a head and neck surgeon, a medical oncologist, and a radiation oncologist. Most teams also have present speech pathologists and access to prosthodontics. Key issues to be determined at initial presentation include staging and resection ability of the tumor [102].

Tumors staged as T1 or T2 with no nodal involvement – early stage tumors - are typically best treated with surgery or radiation depending on the subsites and expertise of the center. Intermediate stage tumors such as infiltrative tumors, negative prognosis and T2 tumors, or exophytic T3, N0-N1 tumors may derive benefit from combined modality treatment. Locally advanced tumors with infiltrative primary sites or regionally advanced cervical disease I best treated with concurrent chemoradiation for unresectable tumors.

For conventional RT of head and neck cancer, the primary tumor and gross adenopathy are irradiated with 70 Gy delivered in single fractions of 1.8-2.0 Gy/d, typically over a six-week period. Significant experimentation with fractionation has been undertaken.

Recent results suggested that her locoregional control with altered fractionation including hyperfractionation (1.2 Gy twice daily; 81.6 Gy over the course of 7 weeks), accelerated fractionation (1.6 Gy daily; 67.2 Gy over the course of 6 weeks), and accelerated fractionation with a concomitant boost (1.8 Gy daily and 1.5 Gy daily as a boost for the last 12 days only; 72 Gy over the course of 6 weeks). A newer and more transform of RT delivery and three-dimensional confirmation fashion is intensity-modulated RT (IMRT), which allows delivery of high doses of RT to clinical target volumes while preserving critical normal structures and salivary gland function.

Dominant site of failure seems to have shifted to distant metastasis as a result of intensive concurrent chemoradiation regimens now widely used for the management of locally past head and neck cancer. Three prospective randomized controlled phase II trials have investigated induction chemotherapy followed by concurrent chemoradiation for the treatment of distant metastasis [104-106]. These trials compared the 3 drug combination of docetaxel, cisplatin, and 5-fluorouracil (5-FU) to the 2 drug regimen of cisplatin and 5-FU as induction chemotherapy. Received is found that survival was significantly better with docetaxel-cisplatin-5-FU (TPF) compared with cisplatin-5-FU. Toxicity appears to be relatively low with TPF. At this point, it has not been proven that induction chemotherapy with TPF followed by chemoradiation is better than chemoradiation alone.

3.4. Concurrent Chemoradiation by Subsites

3.4.1. Oropharynx. The oropharynx is defined as the pharyngeal wall between the pharyngoepiglottic fold and the nasopharynx, soft palate, base of tongue, and tonsil area. Early stage cancer patients (T1-T2, N0-N1) have a 5 year survival rate of 80% to 85%; those with more advanced disease (T3-T4, N0; T3-T4, N+; or any T, N2-N3) have a 5 year survival rate of 30% to 60%. The presence of distant spread reduces this 5 year survival rate to 5% to 10%. Early stage disease can be treated with radiation alone or with radiation and brachytherapy [107].

Concurrent chemoradiation is the standard of care for nonsurgical management of resectable cancers and for unresectable oropharyngeal cancers typical of advanced stage tumors [108-111]. The Groupe d'Oncologic Radiotherapie Tete et Cou (GORTEC) trial reported by Calais et al. [109] and Denis et al. [110] randomly assigned 226 patients with stage III or IV squamous cell carcinoma of the oropharynx to either RT alone (70 Gy in 35 fractions) or RT with concomitant carboplatin and infusional 5-FU. Superior 3 year survival (51% versus 31%; $p=.02$) and disease-free survival (42% versus 20%; $p=.04$) were observed with concomitant chemoradiation versus RT alone. Greater toxicity was noted in the concomitant group [109]. In a later GORTEC study, 163 patients with oropharyngeal and hypopharyngeal cancers received twice-daily radiation versus conventional schedule radiation with cisplatin and 5-FU. Subset analysis of the 123 patients with advanced oropharyngeal cancers illustrated significant better OS for the combined group.

The North American Intergroup trial compared RT alone with RT and a single agent cisplatin and Secours RT with concurrent combination 5-FU and cisplatin [108]. Patient inclusion criteria was limited to patients with unresectable cancer and was not site-specific; oropharyngeal cancer predominated.

It is recommended that patients with advanced resectable disease to undergo concurrent chemoradiation undergo evaluation 8-12 weeks after completion of treatment to detect any residual cancer and to determine the need for neck dissection or salvage surgery of the primary site. This assessment could require the patient to be anesthetized for biopsy and may include biopsies of suspect mucosal abnormalities and imaging of the primary site and neck. The requirement for a planned neck dissection for patients with in 2 or N3 disease at initial staging and to go onto enjoy a complete response remains controversial. The availability of fluorine 18-labeled fluorodeoxyglucose (FDG)-PET with fused CT imaging and correlation with existing trials is expected to yield additional recommendations which will inform this uncertainty.

3.4.2. Hypopharynx. The piriform sinus is the most common site of origination for hypopharyngeal cancers (65%-80%) [111]. Cancer of the hypopharynx tended to be aggressive with diffuse local spread, early nodal metastasis, and a high rate of distant metastasis. Amdur, et al. study a large cohort of patients with squamous cell carcinoma of the larynx and hypopharynx. 87% of patients with piriform sinus primary sites and 82% of patients with posterior pharyngeal wall sites were found to have stage III or IV disease at the time of diagnosis. Laryngopharyngectomy and postoperative adjuvant radiation therapy have been mainstays of treatment. Nonsurgical management is also an option with concomitant chemoradiation therapy.

Early stage hypopharyngeal cancer patients including T1N0 to N1, and low volume T2 N0, have a 5 year survival of 70% to 90%. Those with more advanced disease including stage T2 to T4 and any N, have a 5 year survival of 15% to 30%.

In the setting of early stage disease, definitive RT with surgery for residual neck disease and observation are recommended for patients with complete response [112]. In the setting of more advanced disease, induction chemotherapy and RT are recommended for larynx preservation on the basis of the European Organization for Research and Treatment of Cancer (EORTC) randomized trial [113]. One hundred ninety four patients with stage II, III, IV resectable squamous carcinoma of the piriform sinus (152 patients) and aryepiglottic fold (42 patients) were included in the study. Patients with T1 or N2c disease were excluded. Patients were randomized to laryngopharyngectomy and postoperative RT vs chemotherapy with cisplatin 5-FU for up to 3 cycles followed by RT in those achieving clinical complete response. Equivalent statistical survival was observed in the 2 arms of the study; a functioning larynx was preserved and 42% of patients who did not undergo surgery. Patient who underwent chemotherapy showed a significant reduction in this metastasis as a site of first failure ($P=0.041$).

Posttreatment surveillance of these patients is similar to that of patients with advanced oropharyngeal tumor to undergo concurrent chemoradiation. The examination includes

pharyngoscopy, laryngoscopy with biopsies of suspect areas, and imaging of the primary site and neck 8-12 weeks after completion of treatment. This may require general anesthesia. Salvage surgery to the primary site and neck dissection are indicated that residual disease is detected.

3.4.3. Larynx. Treatment of squamous cell carcinoma of the larynx requires consideration of site (supraglottis, glottis, subglottis) and stage (early, intermediate, locally advanced). Laryngeal voice preservation with a high cure rates are the main goals and treatment of laryngeal cancer with organ-preserving surgery, radiation, or concomitant chemoradiation.

Favorable low volume (<6 mL) supraglottic tumors are usually treated with definitive RT for an open partial laryngectomy, alone or in combination with postoperative RT [114]. Early-stage (T1-T2, N0) tumors of the glottis are treated with either transoral laser excision, RT, or open partial laryngectomy. Literature review shows that the rate of local control, laryngeal voice preservation, and survival are comparable with each of these modalities when patient are appropriately selected [114]. Of note, T2 glottic cancers with paraglottic space invasion were upgraded to T3 status in the 6 edition of the AJCC staging system; this was done because of the poor prognosis due to the risk of local recurrence and treated with radiation therapy alone [98]. Concurrent chemoradiation with the goal of preserving organ function is the mainstay of treatment for locally advanced cancers, including T2B to low-volume T4 tumors.

The Department of Veterans Affairs Laryngeal Cancer Study Group demonstrated in 1990 that that the larynx could be preserved without jeopardizing survival; it compared patients treated with total laryngectomy followed by post-operative RT with those treated with induction chemotherapy followed by RT. This was a landmark study because non-surgical treatment with the goal of curative intent and organ preservation became standard treatment for patients with stage III or IV laryngeal tumors [99]. Over the following decade, research focus shifted from induction chemotherapy to concomitant

chemotherapy and RT as definitive treatment for unresectable cancer and NPC, and for resectable disease in patients preferring organ preservation to initial surgery.

A second major landmark trial was RTOG 91-11. This was a 3-arm, randomized, phase 3 trial enrolling 537 patients [115]. This included patients with stage III or IV (T2 to low-volume T4, M0) glottis or supraglottic laryngeal cancer. Excluded from the trial were patients with T1 lesions and with T4 lesions that penetrated through cartilage or invaded more than 1 cm of the base of the tongue. Patients were randomly assigned to one of three treatment groups: 1) Radiation alone (70 Gy, 35 fractions), 2) concomitant cisplatin and RT, 3) induction cisplatin and 5-FU followed by RT for patients with partial or complete response of the primary site. For all groups laryngectomy was reserved for disease persistence or relapse. At 2 year follow up, the results demonstrated a larynx preservation rate of 88% with concomitant chemoradiation, 75% with induction chemotherapy and radiation, and 70% with RT alone. Locoregional control was also significantly improved with concomitant treatment compared to the other arms (78% vs 61% vs 56%, respectively). This demonstrated the advantage of concomitant over sequential chemotherapy and RT. It is noteworthy that the rates of locoregional control and larynx preservation did not improve with the addition of induction chemotherapy to RT. Similar results were reported at the 5 year follow up [100].

These results indicate that concomitant chemoradiation is the standard of care for advanced laryngeal cancers. Surgery is reserved for patients with persistent disease or recurrent disease after treatment completion.

3.4.4. Treatment: Nasopharynx. Nasopharyngeal cancer is different from other head and neck cancers in multiple facets [116]. They are characterized by a proclivity for local invasion to the cavernous sinus and frequently involve cranial nerves III, IV, and V, and erode through the base of skull. Regional neck disease occurs in 75 to 90% of patients with NPC; bilateral involvement is seen in 50% of patients at the time of diagnosis.

Patients also have a significant risk for distant metastasis, most commonly to bone, lung, and liver.

Three histologic subtypes are recognized in the WHO classification for NPC: Type I, well to moderately well-differentiated SCC which is similar to other head and neck SCC. Type II, non-keratinizing, transitional carcinoma or lymphoepithelioma; and type III, undifferentiated carcinoma. In the United States, the most common type is Type I. In southeast Asia, Type II and III are endemic and are associated with EBV; these types are more radiosensitive and chemosensitive [116].

In the United States, the AJCC TNM staging system is preferred; in Asia, the Ho staging system is favored. Early stage NPC patients (including T1 and T2aN0) have a 5-year survival of 80%. Later stage NPC patients (T2b to T4 and any N) have a 5-year survival varying from 20% to 60%. In the case of distant spread, the 5-year survival rate drops to 5%-10%.

As a result of the US Intergroup nasopharyngeal study, the management of locally advanced NPC has changed during the past decade [100]. For this study, stage III or IV NPC patients were randomly assigned to either RT alone (70 Gy, 35 fractions over 7 weeks) or the RT with 3 concomitant planned doses of cisplatin (100mg/m²) given q21 days, then followed by 3 cycles of adjuvant cisplatin (80mg/m²) and 4 days continuous infusion of 5-FU at 1000 mg/m² per day. The majority of patients (91%) had stage IV disease, and WHO type I histology was more common. With a minimum follow up of 5 years, Overall survival was significantly improved for patients receiving combined-modality treatment: 67% vs 37%, P<.001; this was the case in spite of the fact that only 55% of patients received all three cycles of adjuvant chemotherapy and 5-FU. Significant reduction in the rates of both locoregional and distant failure were noted in patients in the combined-modality arm of the study. The MAC-NPC meta-analysis demonstrated a survival benefit for chemotherapy and combined modality treatment [117]. This result finding remained significant when WHO Type I disease was excluded.

The standard of care for NPC stages IIB, III, IVA (T4N0-N2, M0), and IVB (any T3M0) in the United States is radiotherapy (70 Gy) with concurrent high-dose cisplatin on days 1, 22, and 43 followed by three courses of adjuvant cisplatin and infusional 5-FU, as per the dosing schedule published in the Intergroup trial lead by Al-Sarraf, et al. [100].

3.4.5. Unknown Primary Site HNSCC. Of all new head and neck cancer diagnosed, 2-5% involve an unknown primary source. Typically, these present with a neck mass. In a middle aged or elderly patient with a smoking or alcohol use history, a primary tumor of the head and neck should be suspected. This is also true in the setting of a younger patient without a tobacco history who may have an occult HPV-related cancer of the palatine or lingual tonsil. These patients often have sizable cystic neck masses.

The initial diagnostic test should be a fine needle aspiration of the neck mass. If this is non-diagnostic, it may be repeated or a core-biopsy can be performed. If these tests do not provide a definite diagnosis, excisional biopsy should be performed. This should be done in such a manner that the incision for biopsy can be expanded to accommodate a neck dissection. It is thought that open biopsy can contaminate the surgical field and cause dissemination of malignancy.

When a diagnosis of HNSCC is established in a patient with a cervical lymph node, a systematic and detailed examination of the head and neck under anesthesia is warranted. “Directed biopsies” targeting any suspicious mucosal abnormality and areas known to exhibit high frequency of occult primary lesions. These include the hypopharynx, tongue base, and nasopharynx. Ipsilateral or bilateral tonsillectomy is also indicated given the increasing incidence of HPV-associated tonsil cancers.

Imaging including PET-CT scanning is also recommended in the work up of unknown primary HNSCC and should be considered a standard intervention. The role of PET-CT in this setting has been evaluated in a 16-study meta-analysis in patient presenting with

metastatic cervical lymph node malignancy of unknown primary site. Authors reported an overall sensitivity of 88% and specificity of 75% [118].

In most cases, unknown primary HNSCC is treated with a comprehensive neck dissection involving levels I-V followed by adjuvant RT [119, 120]. Targeting of RT to primary sites will depend on clinical judgement and should be carefully measured against the increased morbidity to be expected with higher doses of radiation. Among patients with high-risk criteria such as positive margins or extracapsular spread, adjuvant chemoradiation should be considered. There has been a trend toward increasing use of combined modality treatment among unknown primary HNSCC patients. The decision to pursue this depends on the care team's belief of likely primary site [121, 122].

3.4.6. Advanced and Recurrent Disease. In spite of numerous advances, aggressive treatment of locally advanced disease yields only 35% to 55% of patients alive and disease free 3 years after standard curative treatment. Thirty to 40% of patients will develop locoregional recurrence and distant metastasis occur in 20% to 30% [115].

Among locoregional recurrences, two major classifications of disease exist: resectable vs non-resectable. Among patients with low-volume disease, treatment with re-irradiation or cyberknife can be recommended with intent to cure. Among patients with unresectable large-volume recurrence or those with distant metastasis, palliative management is recommended, as the disease is considered incurable. Palliative management can take a wide variety of forms, including systemic chemotherapy and biological agents.

Platinum compounds, taxanes, 5-FU, methotrexate, and isosfamide are all potential agents for palliative treatment. Combinations of these agents can yield response rates from 20%-40%. In most cases, the duration of response is in the range of 2-4 months and does not impact overall survival when compared with single agents [123-125].

Recent studies examined the use of cetuximab combined with RT in advanced HNSCC and in recurrent and metastatic disease. In a phase 2 study in patients with recurrent and metastatic disease in which cetuximab was used as a single agent, an objective response rate of 12.6% was observed; the median time of progression was 2.8 months and the median overall survival was 5.7 months [126].

Based on this and other phase 2 studies [127, 128], a phase 3 trial was performed. This examined cetuximab combined with cisplatin in a randomized fashion with 117 patients with either recurrent or metastatic HNSCC. Patients were randomized to either cisplatin plus cetuximab or cisplatin plus placebo [129]. In the study group, the overall response rate was improved (26% vs 10%; $p=0.03$), median PFS (4.2 vs 2.7 mos), and median OS (9.2 vs 8.0 months) when compared to the control group. The treatment group did experience an increase risk of neutropenia, acneiform rash, and dyspnea.

Another trial looked at cetuximab combined with cisplatin and 5-FU. Four hundred forty patients with recurrent or metastatic HNSCC were randomized to either cisplatin-5-FU-cetuximab vs cisplatin-5-FU. Remarkably, the overall survival was greater in the chemo-plus cetuximab group than it was in the chemotherapy alone trial (10.1 months vs 7.4 months, $p=0.036$). It is noteworthy that this is the first demonstration of improvement in OS over the standard cisplatin-5-FU regimen.

It is important to carefully assess performance status when contemplating treatment protocols in the setting of recurrent and metastatic HNSCC. Combination therapy should only be considered in patients with favorable performance status. Those with unfavorable performance status should be treated with single-agent chemo. Unfortunately, patients with bulky locoregional disease and those with high volume of tumor and prior treatment respond poorly to any treatment.

3.4.7. Post Operative Adjuvant Chemoradiation. In spite of advanced surgical techniques and careful patient selection, locoregional recurrence and distant metastasis

can occur after surgery for stage III and IV HNSCC patients. Among patients with positive margins and extranodal spread the risk of recurrence is particularly high [130-132].

Two recent studies serve as the basis for guidelines in the treatment of patients in this situation. These studies – one by EORTC[130] and the other by RTOG [131] – had comparable design. The studies included patients with cancers of the oral cavity, oropharynx, larynx, or hypopharynx who had undergone complete resection of all gross disease; they also had poor-risk pathologic tumor features. They were randomized to postoperative RT alone or to the same RT scheme with concurrent cisplatin on days 1,22,43. In the EORTC study, the 5-year results indicated significant benefit in locoregional control, disease free survival, and OS in the concurrent cisplatin plus RT group. In the RTOG study, preliminary results at 2 years demonstrated improved locoregional control and disease free survival with adjuvant chemoradiation. There was no significant improvement in OS. Both studies demonstrated a greater toxicity with combined therapy. Analysis of pooled data from both studies revealed subsets of patients who benefited from combined treatment included those with either microscopically positive margins or extracapsular spread in neck disease [132]. A long-term 5-year RTOG analysis did not show statistical significance for any endpoint [133]. Subset analysis suggested that patients with positive margins or extracapsular spread as their only risk factor benefit from combination therapy and these are currently considered as a good indication for this treatment.

4.0. REVIEW OF PPARs AND CANCER

4.1. Solid and Hematologic PPAR γ Activity

4.1.1. Hematologic Tumor. Anti-myeloid effects of PPAR γ agonists. A number of anti-cancer mechanisms for PPAR γ have been elucidated. As previously outlined, differentiation therapy has been successfully applied to selected hematologic malignancies. PPAR γ is abundantly expressed in macrophages and myelomonocytic

leukemia cells. PPAR γ ligands induce a variety of anti-cancer effects in acute myelomonocytic leukemia cells, including the differentiation of these cells toward a macrophage phenotype, increased expression of the CD36 scavenger receptors, as well as other surface markers associated with differentiation, including CD11b, CD14, and CD18 [134].

HL-60 cell proliferation was found to be suppressed by troglitazone through G0/G1 cell-cycle arrest and apoptosis. Monocyte differentiation was also induced at the same concentration [135].

Synergistic enhancement of the apoptotic and differentiating effect of troglitazone was achieved with simultaneous treatment with RXR-selective ligands (LG100268); treatment with this agent alone did not produce an apoptotic or differentiating effect.

Fujimura et al. demonstrated arrest of HL-60 cells at the G1 phase and induction of differentiation into monocytes [136].

Yamakawa-Karakide et al. [137] found that PPAR γ activated by the natural and synthetic ligands (15d-PGJ2 and Troglitazone) dramatically inhibited leukemia cell proliferation through preferential induction of apoptosis. They noted that induction of apoptosis was accompanied by caspase-3 activation and was specifically blocked by a caspase-3 inhibitor. C-myc was markedly downregulated within 24 hours after troglitazone treatment, while other apoptosis molecules remained unchanged, thus implicating c-myc as the putative mediator of apoptosis in this cell line. C-myc mRNA levels were dramatically reduced at 1 hour post-treatment and undetectable at 12 hours after treatment. This was associated with complete blockade of the Tcf-4 activity in electrophoretic mobility shift assays. Other studies have demonstrated that PPAR γ ligands can inhibit clonal proliferation of the U937 myeloid monocytic leukemic cells. With the addition of 9-cis-retinoic acid, synergistic inhibition of clonal proliferation of HL-60, U937, and THP1 cells is observed [138].

Eosinophils. EoL-1 is a human eosinophilic leukemia cell line; treatment with TGZ causes cellular arrest at G₀/G₁ phase of the cell cycle; this correlates with upregulation of mRNA for p21^{WAF/CIP1} CDK inhibitor. Inhibition of cell proliferation and expression of p21 mRNA after TGZ treatment was also seen in U937 and in a human myelomonoblastic cell line, KPBM-15. These data suggest that at least one pathway through which TGZ inhibits cell proliferation involves p21 CDK inhibitor [139].

Additional data suggest that RAR- α fusion proteins found in acute promyelocytic leukemia have a negative effect on transactivation of PPAR γ , and that this could be released after treatment with PPAR γ ligands [140].

These data suggest that inhibition of PPAR γ activity may contribute to the pathophysiology of the differentiation block in APL and that PPAR γ ligands could sensitize APL cells to the differentiating effects of ATRA, including ATRA-resistant cells.

4.1.2. Anti-lymphoid Effects of PPAR γ Agonists. PPAR γ anti-cancer effects have been demonstrated in lymphoid malignancies. Both normal murine B cells and multiple B-lymphoma cell lines have been found to express PPAR γ mRNA and protein. PPAR γ activation induces apoptosis [141].

PPAR γ cells have been reported to induce apoptosis in myeloid (U937 and HL-60) cells as well as lymphoid cells (Su-DHL, Sup-M2, Hodgkin's cell lines and primary CLL cells). Combination treatment with PPAR γ ligands and RXR agonists enhance differentiating and growth-inhibitory effects. Exposure to CDDO-induced mitochondrial depolarization and caspase activation was associated with induction of apoptosis [142].

In primary CLL and Jurkat lines, CDDO-induced apoptosis involved caspase-independent loss in mitochondrial membrane potential followed by caspase processing; the pattern suggested that caspase-9 was the apical caspase. CDDO-induced apoptosis

occurred in caspase-8 and FADD-deficient but not in Bcl-xL overexpressing Jurkat cells. In both cell types, CDDO-induced apoptosis primarily by the intrinsic pathway with caspase-9 as the apical caspase [143].

Ray et al. [144] report the novel finding that human multiple myeloma cells express PPAR γ mRNA and protein and that the PPAR γ ligands 15d-PGJ2 and CIZ kill the multiple myeloma cell lines, ANBL6 and 8226, by apoptosis. In addition, the potent myeloma cell growth factor IL-6 did not alter the ability of PPAR γ ligands to kill these cells. Also, the PPAR γ ligands appear to induce apoptosis through mitochondrial damage as measured by a loss in membrane potential and activation of caspases-2 and 3. Experiments also demonstrated expression of RXR by multiple myeloma cells and showed synergistic effects of 9-cis-retinoic acid and PPAR γ ligands for apoptosis induction.

4.1.3. Anti-myeloproliferative Effects of PPAR γ Agonists. Administration of thiazolidinediones is noted and can mottles was accompanied by fat accumulation in the bone marrow cavity and with impaired hematopoiesis [145, 146] resulting in significant anemia. Slight anemia has been recognized as a possible side effect of troglitazone [147]. The lipid accumulation is a result of differentiation of bone marrow stromal cells into fat cells through direct activation of PPAR γ [148]. It is therefore likely that PPAR γ may have a function in diseases such as aplastic anemia; it is possible that PPAR γ may have some therapeutic value in reversing the hyperproliferative state and a myeloproliferative disorder. It has been demonstrated that troglitazone-induced suppression of both proliferation and differentiation of her wrist for a precursor cells in K562 cell; this suggests that activation of PPAR γ could provide a therapeutic method for the treatment of certain types of myeloproliferative disorder such as polycythemia vera.

4.2. Solid Tumor

Physiology and pathophysiology of PPAR function has been primarily explored in the context of adipocyte differentiation, lipid metabolism, insulin sensitization, atherosclerosis and inflammation (see reviews) [149-153].

Additional roles for PPAR γ continue to emerge and significant data support the role for PPAR γ in carcinogenesis in solid and hematologic tumors. The fundamental dysfunction of the balance between cell control and differentiation which underlies malignant cell behavior, including cell proliferation, apoptosis, and terminal differentiation can be expected to be influenced in some part by nuclear hormone receptors.

4.3. Mechanisms of Anti-neoplastic Effects

The induction of differentiation and apoptosis via nuclear hormone receptor ligand activation is a novel approach to cancer treatment. This has been demonstrated with the use of retinoic acids in the treatment of acute promyelocytic leukemia, early lesions of head and neck cancer, squamous cell cancer of the cervix, and skin cancer [32, 154-156].

As many have demonstrated the presence of PPAR γ in a wide variety of both solid and hematologic malignancies. PPAR γ activation leads to inhibition of cell proliferation, apoptosis, and in some cases terminal differentiation. The anti-cancer effects of PPAR γ activation suggest that PPAR γ may represent an effective target for therapeutic gain in the treatment of human cancers.

4.3.1. Mechanisms of Anti-proliferation. Tumor cell line studies have implicated PPAR γ in cell-cycle withdrawal. Cyclin dependent kinase (CDK) inhibitors such as P18, P21, and P27 are targeted by PPAR γ . CDK inhibitors block progression of the cell cycle by inactivating the formation of cyclin/CDK complexes; these complexes are crucial for phosphorylation of retinoblastoma protein when complexed with E2F.

It is generally known that in the non-phosphorylated state, retinoblastoma is a negative regulator of cell-cycle progression. In pancreatic tumor cell lines, cell-cycle arrest in the G1 phase occurs when P21 is induced by glitazones [157].

Upregulation of P21 also occurred in pancreatic cancer cell lines when treated with troglitazone [158]. Upregulation of P27 but not p21 in pancreatic tumors with treatment with PPAR γ agonists was described by Itami and Motomura [159, 160].

Taken together, this data suggests that by upregulation of CDK inhibitors, PPAR γ agonists induce arrest of the cell cycle, though variation among cell types seems evident.

Cell cycle arrest is a second mechanism by which PPAR γ agonists interfere with proliferation. In several tumor cell lines - including those derived from pancreatic cancer, breast cancer, non-small-cell lung cancer (NSCLC), and bladder cancer - cell cycle arrest through inhibition of CDK activity is apparent [21, 31, 161].

In MCF-7 breast cancer cells, troglitazone treatment inhibited cell growth by accumulating cells in the G1 phase. Troglitazone has been shown to target several G1 regulators of pRb phosphorylation, including cyclin D1, CDK2, CDK4, and CDK6. This is particularly strong for cyclin D1. Furthermore, induction of cyclin D1 overexpression partially rescued MCF-7 cells from TGZ-mediated G1 cell-cycle arrest [21]. T24 bladder cell carcinoma cells demonstrated pronounced inhibition of cell proliferation and the presence of cell death as a result of troglitazone treatment. This was caused by an elevation of expression of two CDKI's, p21 and p16, and a concomitant reduction in cyclin D1 expression, in keeping with G1 arrest [162].

Several studies have shown that TGZ targets numerous G1 regulators of pRB phosphorylation, including cyclin D1, CDK 2, CDK 4, and CDK 6, especially cyclin D1. Furthermore, induction of cyclin D1 overexpression partially rescued MCF-7 cells from TGZ-mediated G1 cell cycle arrest [21]. Other researchers have shown that

activation of the PPAR γ receptor has anti-neoplastic effects in Ras-transformed cells. PPAR γ activation resulted in a delay in transit through the G1 phase of the cell cycle; this was associated with inhibition of phosphatidylinositol 3'-kinase (PI3K)/Akt activity as well as a reduction of cyclin D1 expression. PI3K/Akt is an important effector of rest signaling and modulates proliferative signaling. Oncogenic mutations in Ras result and activation of PI3K/protein kinase B [163, 164]. Wortmannin and LY-294002 both dramatically enhance the PPAR activity [165]; this crosstalk between PPAR γ and PI3K pathways is notable and potentially important and may play a significant role in cell cycle arrest and apoptosis. Patel [166] has reported that PPAR γ agonists upregulated expression of PTEN, a lipid phosphatase to place an important role in cell cycle arrest and apoptosis. This is one of the most frequently mutated tumor suppressor gene found in malignancies and can activate P13K.

4.3.2. Mechanisms of Inducing Apoptosis. It has been reported that exposure of human breast cancer cells to a combination of troglitazone and all-trans-retinoic acid decreased bcl-2 protein severely undetectable levels [167]. Comparable results were observed with culture breast-cancer tissues for patient's, but now with normal breast epithelial cells. Zander described in operated leg and in the proapoptotic proteins BAX and BAD as well as a functional role of BAX up regulation for the induction of a proptotic cell death in the context of C6 glioma cells [168]. By the release of cytochrome C. and subsequent activation of several effect or caspases, up regulated expression of BAD and BAX was shown to cause apoptosis. A colon cancer cells, Shimada, et al., noted cell death and apoptosis in colon cancer cells after treatment with troglitazone; this effect was abolished by head caspase inhibitors [169]. In human malignant astrocytoma cells, PPAR γ activation leads to increased caspase 3 activity [170]. Eibl et al. [171] observed that human pancreatic cells treated with PPAR γ agonists exhibited decreased cell viability and growth which was partially mediated by the induction of caspase 3 independent apoptosis, as a specific inhibitor of caspase 3 failed to protect these cell lines from PPAR γ agonists induced apoptosis while at the same time the head caspase inhibitor ZVAD-FMK did. Thyroid is known was found to induce activation of cell death

protease, caspase 3, but not caspase 8 in human liver cancer cell lines. Ohta et al. [172]] observed no increase in BAX protein on treatment with good results in human thyroid carcinoma cells. Shimada et al.[169] failed to identify visible changes in mRNA levels of bcl family genes and HT-29 colon cancer cells after incubation with 15d-PGJ2 or troglitazone. They did find PPAR γ ligand induced apoptosis was associated without regulation of c-myc expression, up regulation of c-jun, and gadd153 expression. They also noted PPAR ligand induced apoptosis was antagonized by signaling mediated through PI3-K. TRAIL, a member of the TNF family cytokines that induces apoptosis, preferentially kill tumor cells wellspring normal tissues. A variety of natural and synthetic PPAR γ ligands sensitize tumor but not normal cells to apoptosis induced by TRAIL. FLICE-inhibitory protein (FLIP) is an apoptosis suppressing protein that blocks early events in TRAIL/TNF family death receptor signaling. PPAR γ ligands selectively reduce levels of FLIP. PPAR γ modulators induced ubiquitination and proteasome-dependent degradation of FLIP, without concomitant reductions in FLIP mRNA [173]. Taken together, these findings suggest that multiple alternative apoptotic pathways can lead to cell death and response to PPAR γ activation.

4.3.3. Mechanisms of Inducing Differentiation. In human primary liposarcoma cells, PPAR γ activation induces in vitro and in vivo terminal differentiation characterized by acute ablation of intracellular lipid and induction of adipocyte-specific genes [174, 175]. Light and activation of this receptor and human breast-cancer causes extensive lipid accumulation and changes in breast epithelial gene expression associated with a more differentiated and a less malignant state [176]. Elnemr et al. [157] demonstrated in breast and pancreatic cancer cell lines, PPAR γ agonists induced up regulation of several differentiation markers such as CEA, E-cadherin, and alkaline phosphatase. PPAR γ activation in malignant rat glioma cells results in apoptosis and induces transient expression of the glioma differentiation marker N-cadherin accompanied by stellate astrocyte-like growth of processes in a small subset of malignant cells [168]. Guan et al. [162] demonstrated in bladder cancer cell line T24 cells, TGZ induced and endogenous PPAR γ target gene, adipocyte-type fatty acid binding protein (A-FABP),

the expression of which correlates with differentiation. Han et al. [177] demonstrated that in LA-N-5 human neuroblastoma cell lines, the natural PPAR γ ligand 15PG-J2, as well as synthetic PPAR γ agonist GW1929, can stimulate the differentiation of neuroblastoma cells; this was evidenced by the inhibition of cell proliferation, neurite outgrowth, increased acetylcholine esterase activity, and the reduction of N-myc expression. The expression of this receptor correlates with the maturational stage of the neuroblastoma cells. Szabo et al. [31] demonstrated in NSCLC cell lines that treatment with PPAR γ ligand in the presence of Sturms resulted in the reversible loss of capacity for anchorage-independent growth, decreased activity and expression of multiple markers in a manner consistent with differentiation. Up-regulation of general markers of differentiated state and down-regulation of nonspecific markers of progenitor lineages for the peripheral lungs were noted. Furthermore, HTI56, a marker of terminally differentiated type I pneumocytes was also induced. These findings demonstrate a shift toward a more mature and less malignant phenotype.

4.3.4. Mechanisms of Inducing Angiogenesis. Angiogenesis is an important biological function which is critical for multiple processes including embryonic development, ovulation, and wound healing. Angiogenesis is also a critical function for solid tumor growth [178] as well as other pathologic conditions such as diabetic retinopathy and age-related macular degeneration. Angiogenesis involves complicated steps which have been well-characterized over the past decade. These include degradation of the basement membrane by cellular proteases, penetration and migration of endothelial cells into the extracellular matrix and endothelial proliferation. This process is controlled by numerous molecules, or factors, most important among them are the vascular endothelial growth factor (VEGF) family of receptors which are the primary mediators of angiogenesis [178].

Solid tumor growth, progression, and metastasis have been shown to be dependent on angiogenesis. Anticancer therapies aimed at angiogenesis have been effective in maintaining long-term remission in patients with advanced metastatic disease that is

resistant to other types of treatment [179] and appears to avoid induction of acquired anticancer drug resistance [180, 181]. In addition to direct PPAR γ driven anticancer effects, evidence has suggested there may also be secondary effects on tumor growth through the regulation of angiogenesis [182]. Barak et al. [183] demonstrated that PPAR γ knockout mice embryos diet on day 10 of life secondary to interference with terminal differentiation patterns of trophoblasts as well as placental vascularization. Additional studies have demonstrated that activation of PPAR γ could inhibit endothelial cell proliferation [182, 184, 185], induce endothelial cell apoptosis [186, 187], inhibit VEGF-induced endothelial cell migration in vitro [184], and inhibit VEGF-induced angiogenesis in vivo [182, 184]. Three important genes for angiogenesis are inhibited by PPAR γ activation: These include the VEGF receptors 1(Flt-1) and 2 (Flk/KDR) and urokinase plasminogen activator (uPA) [182]. In healthy skin endothelial cells as well as tumor endothelium, high concentrations of PPAR γ are present. Activation of PPAR γ can stimulate PPAR γ expression and tumor endothelial cells [185]. Thus it is logical to target tumor endothelium for PPAR γ agonism. One study has demonstrated a decrease in VEGF production in tumor cells through PPAR γ activation [185].

Leptin is a hormone which regulates floor intake and whose receptor (ObRb) has been identified in human vasculature and in primary cultures of human endothelial cells. Both in vitro and in vivo studies have revealed angiogenic activity of leptin, suggesting that the vascular endothelium is a target for this hormone [188]. It is possible that PPAR γ could down-regulation leptin [189] and block left and stimulated endothelial cell migration by inhibition of Akt and eNOS [190]. Analysis of PPAR γ expression in 75 human bladder tumor specimens was compared with clinical and pathological characteristics of the disease. Research is found that PPAR γ was expressed more significantly and papillary tumors and solid tumors, and that its presence was associated with statistically significantly lower incidences of tumor recurrence and progression [191]. Taken together, these observations show that PPAR γ activators are cogent angiogenesis inhibitors in vitro and in vivo and suggest that PPAR γ activators may be an important molecular target and anti-angiogenic therapy.

4.4. Mutations of PPAR γ

The gene for PPAR γ has been mapped to chromosome band 3p25; a number of human malignancies have demonstrated abnormalities at this location. In follicular thyroid carcinoma, PPAR γ is fused to PAX8. The chromosomal translocation t(2;3)(q14;p25) was demonstrated in 5 out of 8 follicular carcinomas of the thyroid; this results in expression of the chimeric fusion protein of PAX8, a thyroid transcription factor, and PPAR γ [192]. Studies have also shown that the PPAR γ gene is functionally mutated in sporadic colon cancer cells [193]. One non-sense and two missense mutations in exon 5 within the ligand binding domain and one frameshift mutation in exon 3, which codes for the DNA binding domain, were identified [194]. These findings inspired the investigation of the expression and mutational status of the PPAR γ gene in a variety of cancer types. Of 397 clinical samples and cell lines, including colon, prostate, breast, and lung cancers, PPAR γ was detectable in all specimens but no abnormality was detectable in any of the human malignancies [195]. This suggests that PPAR γ mutations may occur in cancers, but that they are extremely rare.

4.5. Complexity of PPAR γ Anti-neoplastic Effects

While the previous studies suggest a convincing anti-cancer effect of PPAR γ agonism, data from several authors suggests conflicting findings indicated a more complex relation of PPAR γ to cancer [196-198]. Activation of PPAR γ involves heterodimerization with RXR, the presence of various cofactors, and binding to different PPREs. Studies indicate that the effect of PPAR γ on cell differentiation, proliferation, apoptosis, and inhibition of angiogenesis depends on the cell type in addition to mutational events that predispose tissues to form cancer. Because the bulk of data on PPAR γ has been generated in *in vitro* cell culture conditions, it is difficult to extrapolate to *in vivo* conditions. In two studies evaluating colon cancer treatment of mice using troglitazone, an increase in frequency of colon cancer compared to placebo was observed [199, 200]. *Mim* mice exhibit a germline mutation of the APC gene; this leads to an increase in small and large intestinal tumors. *Mim* mice undergoing treatment developed colon cancers at a ratio of 3:1. In

normal mice, the APC protein binds to beta-catenin, which hold the APC in the cytoplasm and enhances degradation. Mutant APC loses this binding effect; as a result, colon of Mim mice have high levels of beta-catenin, which enters the nucleus, binds to and activates a family of transcription factors called TCF's. This results in the transcriptional activation of a number of cell-cycle-related proteins, including c-myc and cyclin D1 [201, 202]. For uncertain reasons, the treatment of Mim mice with TZDs increases the expression of beta-catenin in colon tissue [200]. Normally, there is a decrease in colon epithelial beta-catenin expression as the tissue undergoes differentiation from the crypts to the tip of the villi; this dampens the growth-promoting effects of TCFs. It appears that Mim mice have a dysregulation of the normal process of differentiation. In humans, mutation of the APC gene is often the initiating event for about 80-90% of sporadic colorectal tumors [203].

In a separate series of experiments, mice were genetically altered such that they constitutively expressed high levels of PPAR γ in their breast tissue. The transgenic mice were mated with mice expressing the mammary tumor virus polyoma middle T which are prone to deep breast cancer. Offspring mice had accelerated kinetic of breast cancer development, indicating that a ligand-activated PPAR γ can under certain circumstances enhance the development of breast cancer [204]. The implication of these two experiments is that once an initiation event has occurred, increased PPAR γ signaling might be capable of promoting tumor progression in selected tissues.

Clinical trials have been performed investigating the anti-cancer effects of PPAR γ ; these have included liposarcoma [174, 205], prostate cancer [206, 207], and refractory breast cancer [208]. Overall, the results of these trials show mixed findings: some demonstrated beneficial effects [174, 206, 207] while others did not [205, 208]. It is noteworthy that neither the hormone status nor the amount of PPAR γ protein was evaluated prior to inclusion of patients in these studies. It is also noted that drug concentration studies of PPAR γ activation in humans has not been studied; it is possible that a sufficient drug concentration for the respective PPAR γ agonist may not have been

achieved by oral administration. It is possible that PPAR γ treatment may be more useful in a chemopreventative function or may be effective as an adjuvant therapy for conventional chemotherapy or radiation therapy. At present the number of human clinical studies is limited; however, the initial results do justify further investigation.

5.0. PPAR γ and CA

5.1. Colorectal Cancer

Drugs exerting an anti-inflammatory action could hinder the proliferation of tumor cells, especially in the case of colon cancer [209]. Not all of human colon carcinoma cell lines that express PPAR γ , and ligand activation of this receptor caused most, differentiate a response to overturn their malignant phenotype [48, 59]. There is an abundance of PPAR γ expression in the intestine, especially in the colon, indicated by quantization of PPAR γ by reverse transcription-polymerase chain reaction (RT-PCR) in various different types of tissues. This may indicate that PPAR γ could be important in the human colon, under both pathological or normal circumstances [210]. Ornithine decarboxylase activity and colonic mucosa polyamine content was decreased while apoptosis were increased by TGZ treatment in a rat model stimulated with abnormal crypt foci from precursor lesions for colon carcinoma, azoxymethane (AOM) treatment. Abnormal crypt foci formation by AOM and dextran sodium sulfate (DSS) induced colitis were inhibited by gastric gavage of TGZ. According to this data, when colitis is present, it is suggested that PPAR γ ligand used may inhibit the premature stages of colon tumorigenesis [211, 212]. HT-29 colon cancer cells were screened with cDNA arrays examining the modulation of apoptosis related gene expression by PPAR γ ligands and confirmed by quantitative RT-PCR analysis. There were not any considerable effects of ligands for PPAR δ or PPAR α . The PPAR γ ligands 15d-PGJ2 and TGZ suppressed DNA synthesis of HT-29 cells. HT-29 cell death was induced with both PPAR γ ligands used in a dose dependent manner; which also was susceptible to a caspase inhibitor and an increase in fragmented DNA. Quantitative RT-PCR analysis confirmed down- regulation of c-myc expression and up-regulation of c-jun in many genes selected by cDNA array screening. Quantitative RT-

PCR analysis also confirmed growth arrest DNA damage (GADD) inducible 153 gene expression by 15d-PGJ2 and TGZ. Mutated APC that caused deregulated c-myc expression seemed to be balanced by the activation of PPAR γ signaling, even as c-myc is an important target gene of the APC/beta-catenin and/or APC/gamma-catenin pathway [169]. After the ciglitazone treatment, HT-29 human colon cancer cells were also induced with apoptosis [213].

Different ways that PPAR γ can control intestinal epithelial cell biology were emphasized by microarray technology and the identification of PPAR γ gene targets in intestinal epithelial cells [214]. This was done by using two different PPAR γ agonists, different by structure, in which the induction or repression change detected by each gene was blocked by a particular PPAR γ antagonist co-treatment. According to this study, PPAR γ selective targets consist of genes related to growth regulatory pathways (regenerating gene IA), colon epithelial cell maturation (GOB-4 and keratin 20), and immune modulation (neutrophil-gelatinase-associated lipocalin). The carcinoembryonic antigen (CEA) family also has three different genes induced by PPAR γ . PPAR γ may be able to control intracellular adhesion. This was shown by cultured cells that were treated with PPAR γ ligands displayed an increase in Ca²⁺-independent and CEA-dependent homotypic aggregation [214].

The synthesis of prostaglandin from arachidonic acid is catalyzed by the enzyme cyclooxygenase and is also the rate-limiting step; COX-2 is the inducible isoform of cyclooxygenase. Different prostaglandins are made in a cell type-specific way and extract cellular functions by signaling through G-protein coupled membrane receptors, sometimes through PPARs [215, 216]. Cellular functions may be affected by COX-2 making use of arachidonic acid and using the free arachidonic acid. Cell and animal models alike have shown that COX-2 may encourage cell growth, improve cell motility and adhesion, and hinder cell death. Not known are the mechanisms to the multiple actions of COX-2 but strong evidence from studies, clinical and genetic, suggests that an important step in carcinogenesis is the upregulation of COX-2. In animal models, if the

COX-2 pathway is inhibited, the result is a reduction in tumor occurrence and progression while if the COX-2 pathway is overexpressed, the result is tumorigenesis [216]. Recently developed COX-2 specific inhibitors and the prospective application of NSAIDs have gained remarkable attention. A worthwhile approach in prevention and treatment of cancer, including but not limiting colorectal, will be the inhibition of COX-2 [216].

PPAR γ has been shown to stimulate COX-2 expression by up-regulation of the TNF-alpha pathway. PPAR α and TNF-alpha signaling increase COX-2 by independent pathways [217]. Colorectal cancer may be produced by heightened levels of 15-lipoxygenase-1 (15-LO-1) found in human colon tumors. 15-LO-1 metabolites could either increase mitogenesis by the up-regulation of epidermal growth factor (EGF-R) signaling pathways or reverse their malignant phenotype by a differentiative response caused by ligands for PPAR γ activation. HCT-116 cancer cells were also inspected for the function that 15-LO-1 and its metabolites had on EGF-R signaling and PPAR γ activation in which exogenously added 15-LO-1 enhanced the PPAR γ phosphorylation and up-regulated the MAPK signaling pathway [68]. In steady HCT-116 cells that overexpress 15-LO-1 and produce endogenous 15-LO-1 metabolites, PPAR γ phosphorylation and an up-regulation MAPK was observed which was annulled when a MAPK inhibitor was applied. The outcome of the down-regulation of PPAR γ activity is caused by the 15-LO-1 up-regulating MAPK activity and an increase in PPAR γ phosphorylation. Consequently, 15-LO-1 metabolites can both down-regulate PPAR γ activity by the MAPK signaling pathway and be ligands for PPAR γ [218].

Several cellular functions are altered by the phosphatase and tensin homologue mutated on chromosome ten (PTEN) tumor suppressor gene, including: cell migration, survival, and proliferation by antagonizing PI-3K-mediated signaling cascades [219]. The Caco2 colorectal cancer cells, along with other tumor cell lines, had upregulated PTEN expression from PPAR γ activation by rosiglitazone. Reduced phosphorylation of protein kinase B allowed the measurement between the correlation of the upregulation and

decreased PI-3K activity; the effect being reduced proliferation rate of Caco2 cells [166]. The upregulation of PTEN was inhibited by the antisense-mediated disruption of PPAR γ expression. A role of PPAR γ , according to these data, is the regulation of PI-3K signaling by modulating PTEN expression in tumor-derived cells [166].

5.2. Gastric Cancer

PPAR γ mRNA and protein were expressed in the human gastric cancer cell line MKN45. Luciferase assay in the human gastric cancer cells showed that TGZ transactivated the transcription of PPRE-driven promoter and TGZ, or pioglitazone treatment, inhibited the growth of MKN45 cells in a dose dependent manner. Induced apoptosis, shown by DNA ladder formation, occurred by co-incubation of MKN45 cells with TGZ [220]. The PPAR γ expression in human gastric cancer, expressed PPAR γ mRNA and protein were both delineated in studies. Immunohistochemical detection of PPAR γ protein in surgical specimens of gastric adenocarcinoma was noted. PPAR γ expression was also demonstrated in gastric carcinoma cell lines. Treatment of these cell lines with TGZ and PGJ2 resulted in dose-dependent inhibition on proliferation. This effect was increased with co-incubation with 9-cis retinoic acid, which is a known RXR-alpha agonist. Analysis with flow cytometry showed G1 cell cycle arrest and an increase in annexin V-positive cells following TGZ treatment. The summary of these results indicates that apoptosis induction along with G1 cell cycle arrest may represent mechanisms affecting the anti-proliferative effects observed in gastric cancer cells [221]. Colonization with *Helicobacter pylori* (HP) results in epithelial cell hyperproliferation in regions of inflamed mucosa; levels of apoptosis are variable in these areas. This suggests that imbalances between rates of cell loss and replacement contribute to differences in gastric cancer risk rates in different HP infected populations. It has been established that PPAR γ affects inflammatory and growth responses of intestinal epithelial cells. In gastric epithelial cell lines sensitive to HP-induced apoptosis, PPAR γ was shown to be expressed and functionally active. Ligands of PPAR γ , including 15-PGJ2 and rosiglitazone significantly attenuated HP-related apoptosis, an effect that was reversed with co-incubation with the specific PPAR γ antagonist GW9662 [222]. Cyclopentanone

prostaglandins that have neither binding nor activation of PPAR γ had no effect on HP-related apoptosis in this setting. Co-treatment of cells with PPAR γ agonists inhibited the ability of HP to activate NF-KappaB target interleukin (IL)-8. NF kappaB was also directly inhibited with the observation of elimination of HP-induced apoptosis. This suggests that PPAR γ pathway activation attenuates the ability of HP to induce NF-KappaB-mediated apoptosis in gastric epithelial cells [222]. The authors of these studies conclude that PPAR γ regulates many host responses and that activation of this receptor may contribute to varying levels of cellular replication as well as diverse pathologic results associated with chronic HP colonization [222].

5.3. Hepatic Cancer

PPAR γ has been shown to be constitutively expressed in all hepatoma cell lines, including HLF, HepG2, HuH-7, HAK-1B, HAK-5, KYN-1, and KYN-2, as well as in hepatocellular carcinoma (HCC) tissue [223, 224]. Among these cell lines, a dose dependent cytostatic effect with TGZ was noted, resulting from the G0/G1 cell cycle arrest; this was felt to be related to triggering of the p21 protein expression. The cell line HLF, which is known to be deficient in pRB expression, responded most to TGZ, demonstrating an increase in expression of p21, p27, and p18. This suggests that p21, p27, and p17 may be connected to TGZ-related cell cycle arrest in human hepatoma cells [223]. Marked growth inhibition was caused in cell lines HepG2, HuH-7, KYN-1, and KYN-2 when incubated with TGZ, pioglitazone, and 15d-PGJ2. This growth inhibition was associated with dose-dependent inhibition of DNA synthesis, cell cycle progression, and alpha fetoprotein expression [225].

Liver cancer cell lines PLC/PRF/5, HepG2, and JuJ-7 were exhibited growth inhibition by TGZ through the induction of apoptosis through caspase 3 activation; this supports evidence that TGZ could be used as an apoptosis inducing agent for HCC [226].

Ciglitazone and 15d-PJ2 both significantly reduced platelet-derived growth factor (PDGF)-induced proliferation in activated human hepatic stellate cells (HSCs) and inhibited alpha smooth muscle actin expression during HSC trans-differentiation.

Interestingly, while treatment with 9-cis-retinoic acid and LG268 – both ligands of RXR – had no meaningful effect in PDGF-treated cells, they caused a further reduction of proliferation when co-treated with ciglitazone [227].

5.4. Pancreatic Cancer

Treatment with TZD resulted in inhibition of both cellular and clonogenic growth and G₀/G₁ cell cycle arrest in various human pancreatic cancer cell lines, including Capan-1, AsPC-1, Capan-2, HPAF-II, BxPC-3, PANC-1, and MIA PaCa-2 [157, 171]. Treatment with TZD resulted in induction of P21 induction and increases in differentiation marker expression [171]. 15-PGJ₂ and Ciglitazone treatment decreased the cell number, viability and increased floating/attached ratios in a time- and dose-dependent manner among six pancreatic cell cancer lines [171]. In part, these effects were mediated by caspase-3 independent apoptosis.

The impact of the administration of TGZ to different cell lines of pancreatic cancer has been examined [158]. PANC-1 cell lines treated with TGZ showed the induction of G₁ phase accumulation with an increase in p27 but not p21 expression and inhibition of cell proliferation. Nude mice implanted with PANC-1 tumors and treated with pioglitazone demonstrated growth inhibition of tumors. A powerful dose-dependent response of TGZ with respect to growth inhibition was shown in 6 or nine pancreatic cancer cell lines which were suppressed less than 50% of control with concentrations of >10M. This observed growth inhibition was associated with the G₁ phase cell cycle arrest through upregulation of p21 mRNA and protein expression; at the same time, inhibition of CDK2 kinase activity and hypophosphorylation of pRb was noted [159]. The duct structure of pancreatic cells was changed with apoptotic cell in the lumen of these treated animals.

5.5. Lung Cancer

Analysis of non-small cell lung cancer (NSCLC) cell lines has revealed PPAR γ mRNA and protein, with increased levels seen in adenocarcinomas. Using immunohistochemistry, 50% of primary lung cancer specimens were shown to contain

PPAR γ protein (n=39) [31]. In another series, among 147 cases of NSCLC, PPAR γ positivity was prominent in 47% of squamous cell lung cancer cases; this was positive in 35% of lung adenocarcinoma cases. Tumors with high histological grades and histological types were most frequently observed to be PPAR γ positive [228]. Among higher differentiated lung cancer cases, PPAR γ mRNA levels were increased. In NSCLC cases, low PPAR γ mRNA correlated with worse survival for patients[229]. Ciglitazone and 15d-PGJ2 treatment of cell lines in normal serum resulted in growth arrest, irreversible loss of capacity for anchorage-independent growth, decreased activity and expression of matrix metalloproteinase 2, and modulation of differentiation markers. After PPAR γ treatment, HT156 – a marker of terminally differentiated type I pneumonocytes was induced. Ligand treatment also inhibited the expression of cyclin D1 and led to hypophosphorylation of the pRb. Without serum in the culture media, treatment with ligands quickly caused apoptosis and substantially earlier onset of differentiation. Cellular growth and apoptosis induction of NSCLC cells in a time- and dose-dependent fashion was seen with treatment with TGZ and pioglitazone. Using subtraction cloning techniques, it was shown that TGZ stimulated expression of the GADD153 gene. Increased expression of GADD153 mRNA was confirmed by array analysis of apoptosis genes. TGZ failed to stimulate GADD153 mRNA expression among cells lacking PPAR γ expression; interestingly, inhibition of GADD153 gene expression was noted when an antisense phosphorothionate oligonucleotide (which is known to attenuate the TGZ-induced growth inhibition) was used. These results suggest that GADD153 may be a factor implicated in growth inhibition and apoptosis, induced by TGZ, via PPAR γ activation [230]. Treatment with TGZ and 15d-PGJ2 inhibited growth of human lung cancer cells through the mechanism of apoptosis; this result was abrogated by application of the PPAR α agonist bezafibrate [23]. When NSCLC cell lines were treated with the combination of histone deacetylase inhibitors (HDAC) and PPAR γ ligands, enhanced growth inhibitory capacity in adenocarcinomas compared to single treatment [231].

5.6. Breast Cancer

Normal lactating breast tissue is known to express PPAR γ , which may regulate mammary epithelial and stromal cell function [232]. Human primary and metastatic breast carcinoma specimens were also shown to contain PPAR γ protein. PPAR γ ligand activation in breast cancer cell cultures caused extensive lipid accumulation, changes in breast epithelial gene expression associated with increased differentiation and less malignancy, as well as a reduction in growth rates and clonogenic capacity. MAPK inhibition is known to be a strong negative regulator of PPAR γ and improved TZD sensitivity in nonresponsive cells. These results suggested that the PPAR γ pathway may induce terminal differentiation of malignant breast epithelial cells and may represent a novel nontoxic therapy for breast cancer [176]. Four days of treatment with TGZ involving MCF-7 breast cancer cells resulted in reversible inhibition of growth. Combined treatment with retinoic acid (RA) and TGZ irreversibly inhibited growth and induced apoptosis; this was associated with a pronounced decrease of their bcl-2 protein levels [167]. When cultured breast cancer tissues from patients were treated *in vitro*, similar effects were noted. In triple immunodeficient mice, TGZ significantly inhibited MCF-7 tumor growth. Co-administration of RA and TGZ resulted in prominent apoptosis and fibrosis of these tumors sans evidence of toxicity [167]. PPAR γ treatment was associated with an anti-proliferative effect in several human breast cancer cell lines. Some differences among cell lines were noted with respect to DNA binding by endogenous PPAR γ in gel shift assays and activation of PPAR γ by prostanoid and TZDs in reporter gene assays. Altogether, these results indicated a variable, cell-specific response to various PPAR γ activators, suggesting a role for PPAR γ related treatment of breast cancer [233].

PPAR γ activation with 15d-PGJ2 or TGZ was shown to attenuate cellular proliferation of the estrogen-receptor negative breast cancer cell line MDA-MB-231, as well as of the estrogen receptor-positive cell line MCF-7. This was associated with a decrease in total cell number as well as an inhibition of cell cycle progression and induction of apoptosis. This suggests that apoptosis may be the primary biological response due to PPAR γ

activation among some breast cancer cells [234]. Multiple researchers have shown that the terminal derivative of prostaglandin 15d-PGJ2 caused apoptosis induction in breast cancer cells; this compound is a known powerful activator of PPAR γ . Early gene expression critical to apoptosis was regulated by 15d-PGJ2. This compound induces powerful and irreversible S phase arrest that correlates with the expression of genes critical to cell cycle arrest and apoptosis. This includes the CDKI p21. RNA and cell protein synthesis inhibition abrogated apoptosis induced by 15d-PGJ2 in breast cancer cells but potentiated apoptosis induced by TNF- or CD95/Fas ligands. In addition, 15d-PGJ2 treatment induced caspase activation that was blocked by inhibitors of peptide caspase [235]. These results indicate that de novo gene transcription is required for 15d-PGJ2-related apoptosis in breast cancer cells [235]. Research using multiple cancer cell lines, animal models and with several PPAR γ activators have shown conflicting results; these include changes in cellular proliferation, differentiation and apoptosis of cancer cells/tumors. The impact of low-, moderate-, and high-dose PPAR γ ligands (including 15d-PGJ2 andn TGZ) on measures of cell growth, differentiation, and apoptosis in epithelial breast cancer cell line MDA-MB-231 were investigated [236]. Researchers showed that the biologic effects of these agents depended significantly on ligand concentration and the degree of PPAR γ activation. 15d-PGJ2 was shown to be a more potent activator than TGZ based on transcriptional activation studies. Overall, however, low transcriptional activation correlated with increased cellular proliferation; higher levels of activation went along with cell cycle arrest and apoptosis [236]. In animal studies, TGZ treatment of tumors induced stasis or regression of total tumor volume in 40-50% of animals vs. only 10% of controls and 65% of tamoxifen-treated animals. Researchers also found that TGZ was as effective as tamoxifen in the prevention of additional tumor development; however, it was not able to reduce the development of malifnant tumors when administered prior to detection [237]. MCF-7 cell line proliferation was inhibited by TGZ by blocking critical events for G1/S phase progression. Cells were found to accumulate in G1 and were accompanied by an attenuation of pRb phosphorylation associated with decreased cyclin-dependent kinase (CDK)4 and CDK2 activity. TGZ was shown to inhibit CDK activity, and this correlated

with decreased protein levels for several G1 regulators of pRp phosphorylation. Overexpression of cyclin D1 partially rescued MCF-7 cells from TGZ-mediated G1 cell-cycle arrest [21]. The biosynthesis of estrogen is catalyzed by aromatase cytochrome P-450, which is a product of the CYP19 gene. Aromatase is mostly expressed in the mesenchymal stromal cells and represents a marker of the undifferentiated preadipocyte phenotype in humans. In pre-menopausal women, adipose tissue is the major site of estrogen biosynthesis. The local production of estrogen in breast adipose tissue is implicated in the development of breast cancer. This has been demonstrated by the measurement of aromatase activity and by RT-PCR/Southern blotting that both synthetic and natural PPAR γ activators TGZ, rosiglitazone, and 15d-PGJ2 respectively, inhibited aromatase expression in cultured breast adipose stromal cells stimulated with oncostatin M or TNF-alpha in the presence of dexamethasone; a dose-dependent relationship was observed [238]. Also noteworthy was that a metabolite of TGZ that is known not to activate PPAR γ had no effect on growth inhibition of the cultured cells [238].

In MCF-7 cells, 15d-PGJ2 mediated activation of PPAR γ resulted in robust inhibition of Erb-2 and Erb-3 tyrosine phosphorylation induced by neuregulin-1 and -2. Also noted was efficient blocking of Erb's with respect to proliferation, differentiation, and cell death. Upon treatment with 15d-PGJ2, prior addition of neuregulins resulted in a dramatic growth-suppressive effect accompanied by G0/G1 phase cell accumulation and a spike in the apoptosis rate [239]. Tumor incidence, tumor number, and tumor weight were all reduced by the synthetic PPAR γ ligand GW7845 when used in the classic rat model of experimental mammary carcinogenesis induced by nitrosomethylurea [240]. The ability of TGZ with or without retinoid co-treatment to prevent preneoplastic lesion induction by 7,12 dimethylbenz[a]anthracene (DMBA) in a mouse mammary gland organ culture model; this supports evidence that TGZ exerted chemopreventative activity [241]. This activity appeared to be enhanced by a ligand for RXR – LG100068 [241].

5.7. Ovarian Cancer

Aromatase activity in cultured human ovarian granulosa cells from pre-ovulatory follicles was decreased by TGZ treatment [242]. Similar findings were observed with treatment of a human ovarian granulosa cancer cell line. Both experiments showed an even greater magnitude of reduction when treatment combined the specific RXR ligand LG100268 [243].

5.8. Prostate Cancer

Untreated prostate cancer cell lines LNCaP, DU145, and PC-3 have been shown to express PPAR γ ; 15d-PGJ2 treatment in these cell lines increased expression of PPAR γ 2 levels while PPAR γ 1 level remained unchanged [244]. When PC-3 cells were cultured with TGZ, dramatic morphological changes by light and electron microscopy occurred. This suggests that the cells were less malignant, however molecular markers of cell cycle and differentiation were not changed [20]. Selective necrosis of tumor cells but not adjacent normal cells was observed in human prostate cancer cells treated ex vivo with TGZ. Oral administration of TGZ in patients harboring advanced prostate cancer without signs of metastatic disease resulted in a high rate or prolonged stabilization or prostate-specific antigen (PSA) and a dramatic decrease in serum PSA to nearly undetectable levels [206]. Three PPAR γ activators, including TGZ, pioglitazone, and 15d-PGJ2 all down-regulated androgen-stimulated reporter gene activity in LNCaP cells. The PSA promoter contains androgen receptor response elements (AREs). Reporter gene studies have demonstrated inhibition of androgen activation of the AREs in the PSA region of regulation. TGZ treatment of LNCaP cells dramatically suppressed PSA protein expression but did not suppress expression of the androgen receptor, indicating that TGZ inhibited ARE activation by an AR independent mechanism [207]. In normal and benign prostate hyperplasia, very weak or no expression of immunoreactive PPAR γ was identified. In contrast to this, in prostate cancer and prostatic intraepithelial neoplasia cases, significant expression of immunoreactive PPAR γ was identified; this suggests the participation of PPAR γ in prostate cancer development and treatment [245].

5.9. Renal Tumors

Human renal cell carcinoma (RCC) specimens have been shown to express PPAR γ protein. RCC cell lines have also been demonstrated to express PPAR γ mRNA and protein. Both synthetic and endogenous ligands have been shown to inhibit growth of RCC cells. Further research is ongoing [246].

5.10. Urinary Bladder

PPAR γ mRNA and protein have been documented as being expressed in several urinary cancer cells, including a non-neoplastic urothelial cell line (1T-1), a low-grade carcinoma cell line (RT4), and two high-grade cell lines (T24 and 253J). Several PPAR γ agonists including 15d-PGJ2, TGZ, and pioglitazone suppressed the growth of non-neoplastic and neoplastic urothelial cell in a dose-dependent manner [247]. Neoplastic cell lines were more resistant than non-neoplastic cell lines; this suggests that the failure of cells to express PPAR γ or transcriptional malfunction may contribute to resistance to the PPAR γ ligand inhibitory effect. Cell proliferation in T24 cells was dramatically inhibited with TGZ treatment and cell death was observed [162]. These effects were associated with an increase in expression of two CDKIs, p21 and p16, as well as reduced cyclin D1 expression, in keeping with G1 phase arrest. TGZ incubation caused the production of the endogenous PPAR γ target gene, adipocyte-type fatty acid binding protein (A-FABP); the expression of this protein correlates with bladder cancer differentiation.

Immunohistochemical analysis of 75 human urinary bladder tumor specimens for PPAR γ expression with correlation of clinicopathologic characteristics as well as molecular expression of molecules related to angiogenesis and cell cycle progression. PPAR γ was expressed at higher levels in papillary compared to solid tumors. PPAR γ was associated to low incidence of tumor recurrence and progression in a statistically significant manner. When PPAR γ was co-expressed with platelet-derived endothelial cell growth factor (PDEC GF), a significant association was also noted. PDEC GF is felt to be an angiogenic

growth factor and is related to poor prognosis. When combined with basic fibroblast growth factor (bFGF), however, PPAR γ expression did not result in a significant association with low incidence of tumor recurrence or progression. These findings suggest a possible worsening role of this angiogenic factor in its interaction with PPAR γ [191].

5.11. Sarcoma

In each of the major histologic types of human liposarcoma, PPAR γ was expressed at high levels. Primary liposarcoma cells treated with the PPAR γ ligand pioglitazone are induced to undergo terminal differentiation [248]. Accumulation of intracellular lipid, induction of adipocyte-specific genes, and withdrawal from cell cycle all were observed as indicators of liposarcoma cell differentiation. In three patients with intermediate to high-grade liposarcomas who were treated with TGZ, induction of histologic and biochemical differentiation of tumors were observed *in vivo*. Biopsies of these tumors revealed histologic evidence of extensive lipid accumulation and substantial increases in NMR-detectable tumor triglycerides compared to pre-treatment biopsies. Several mRNA transcripts typical of differentiation in the adipocyte cell lineage were induced. A marked reduction in tumor proliferative capacity was noted, as evidenced by Ki-67 IHC expression [175]. The impact of PPAR γ activator treatment, including pioglitazone and 15d-PGJ₂, on the chondrosarcoma cell line OUMS-27, has been investigated [249]. Both of these compounds inhibited the proliferation of OUMS-27 cells by induction of apoptotic signals; this was a dose-dependent phenomenon. Interestingly, TGZ did not induce apoptosis in PPAR γ expressing cell lines G292, MG63, SAOS, U2OS, but increased cell number in these cell lines. It was determined that this increase in overall cell number was due to a decrease in apoptotic cell death rather than an increase in proliferative rates. This survival effect mediated by TGZ was correlated with the activation of Akt, a well-known mediator of survival stimuli [196].

5.12. Hematologic Malignancies

PPAR γ has been implicated in the macrophage lineage and plays a role in the regulation of inflammatory responses, including atherosclerosis [250, 251]. Myeloid leukemia cell lines contain PPAR γ protein [138]. By itself, TGZ failed to induce differentiation in any of the cell lines. It did, however, suppress the clonogenic growth of myeloid leukemia cell lines. Myelomonocytic U937 cells were the most responsive to the growth suppressing effects of TGZ. These cells were arrested in the G₁ phase of the cell cycle when cultured with TGZ. Co-treatment of myeloid leukemia cell lines with TGZ and the RXR specific ligand LG100268 or the RAR ligand ATRA and ALART1550 resulted in synergistic clonal growth suppression [138]. BRL49653, a PPAR γ ligand, enhances 9-cis-retinoic acid-related growth suppression on human monocytic leukemia cells, THP-1 cell line [252]. Pioglitazone and TGZ dramatically suppressed cell proliferation of the promyelocytic leukemia cell line HL60, causing a G₀/G₁ cell cycle arrest in addition to apoptotic effect [136, 253]. Treatment with TGZ induced monocytic differentiation of HL60 cells. These apoptotic and differentiating effects were additively enhanced by the co-treatment with LG100268; LG100268 treatment alone had neither an apoptotic nor a differentiating effect on these cells.

Cell proliferation and the erythroid phenotype of K562 cells were suppressed with TZD treatment. Synergistic enhancement of this effect occurred with the combined treatment of with the RXR selective ligand, LG100268. This suppression of erythroid phenotype in K562 cells was associated with the down-regulation of the erythroid lineage-transcription factor, GATA-1 [254]. THP-1, a monocytic leukemia cell line, differentiates to macrophages in response to phorbol 12-myristate 13-acetate (PMA). It was used in the investigation of the 9-cis retinoic acid regulation of genes in the scavenger receptor type B family (CD36) in human monocyte macrophages. CD36 was induced by the natural PPAR γ ligand, 15d-PGJ₂. In addition, the combination of 9-cis retinoic acid and 15d-PGJ₂ further enhanced CD36 protein and mRNA levels over that observed with both compounds independently. PPAR γ antagonism with GW9662 was used to block PPAR γ induction of CD36 gene expression, but had little effect on the action of retinoic acid

[255]. In normal human B cells and a variety of B lymphoma cell lines, PPAR γ protein was expressed. In these cell lines, 15d-PGJ2 and TZDs caused cytotoxicity in these cell lines through apoptosis induction [256].

5.13. Neurological Neoplasms

PPAR γ expression has been observed in 95% of human gliomas of differing pathological stages. Of nine cell lines derived from malignant glioma (SK-MG-1) or neuroblastoma (NB-1), two were PPAR γ positive. In these two cell lines, PPAR γ treatment using TGZ induced growth inhibition, which, upon further investigation, was shown to be mediated by PPAR γ directed induction of apoptosis [257]. Abundant expression of PPAR γ protein was observed in human primary astrocytes and in human malignant astrocytoma cells of the T98G line. Apoptotic cell death resulted from treatment of these cells with 15d-PGJ2 or ciglitazone [170].

15d-PGJ2, GW1929, and phenylacetate (PA) – all PPAR γ ligands – induced neuroblastoma cell LA-N-5 cells to differentiate to a common phenotype; this was manifested by inhibition of cell proliferation, neurite outgrowth, increased acetylcholinesterase activity, and decreased N-myc gene expression [258]. All of these molecular and functional effects were inhibited when the specific PPAR γ antagonists were used.

Inhibition of proliferation and cell death induction in human and rat glioma cell lines were observed when they were treated with the PPAR γ ligands ciglitazone, LY171 833, and 15d-PGJ2. These compounds induced cell death which was characterized by DNA fragmentation and nuclear condensation. It was also inhibited by the synthetic receptor-antagonist bisphenol A diglycidyl ether (BADGE). Interestingly, primary murine astrocytes were unaffected by PPAR γ agonist treatments [168]. Glioma cell apoptotic death following treatment with PPAR γ ligands was associated with the transient up-regulation of Bax and Bad protein levels. Inhibition of expression of Bax by specific anti-sense oligonucleotides had a protective effect against PPAR γ mediated apoptosis;

this indicates an essential role of Bax in PPAR γ mediated apoptosis. Also, PPAR γ ligands caused re-differentiation as implied by the increase in expression of the differentiation marker, N-cadherin [168].

5.14. Thyroid Neoplasms

Immunoreactivity to PPAR γ was found in neoplastic moreso than in adjacent normal thyroid tissue in papillary thyroid carcinoma tumors. 15d-PGJ2, TGZ, and BRL49653 all induced apoptosis in PPAR γ expressing thyroid carcinoma cell line, including BHP2-7,7-13, 10-3, and 18-21. Treatment with TGZ significantly increased c-myc but not bcl-2 and bax mRNA levels in thyroid carcinoma cells *in vitro*. TGZ treatment also inhibited tumor growth and prevented distant metastasis of BHP 18-21 tumors in nude mice, *in vivo* [172].

6.0. NUCLEAR HORMONE RECEPTORS IN HNSCC

Because nuclear receptors are involved in cell proliferation, apoptosis, invasion and migration, and cell cycle regulation, they represent a logical target receptor for cancer therapy. Several highly effective cancer drug therapies target this receptor family [259-262]. Because nuclear hormone receptors are known to be expressed in HNSCC cells, there is a possibility that they are participating in cancer development and progression [263, 264]. The majority of clinical studies and reports in HNSCC have focused on thyroid hormone receptor-like receptor and the estrogen receptor-like receptor [265].

6.1. Thyroid Hormone Receptor-like Receptors

Retinoic acid receptors. A subfamily of thyroid hormone-like receptors, retinoic acid receptors (RAR) subtypes RAR α , β , and γ are each characterized by their ability to be activated by the binding of retinoic acid [259, 266]. Once activated, RARs heterodimerize with retinoid X receptors (RXRs), which belong to the retinoid X receptor-like receptor subfamily; as such, they bind to specific HREs to regulate the

transcription of target genes. A wide variety and number of coactivator- and corepressor-proteins are involved in this regulatory system; this allows transcriptional alteration and tuning ranging from repression to complete activation [267, 268]. Activation of RAR can lead to differentiation, cell-cycle arrest, or apoptosis; it can counteract cell proliferation and tumor progression [268]. For this reason, the ligand retinoic acid and its analogues are under investigation as therapeutic agents in several tumor entities alone and in combination with chromatin modulating agents, such as histone deacetylase inhibitors [259, 268-270].

Malignant tumors of the head and neck have been observed to have reduced RAR β mRNA levels, but appear to be prominent early in premalignant oral lesions [266, 271, 272]. These results were confirmed on the protein level by immunohistochemistry which demonstrated the different RAR $\alpha/\beta/\gamma$ expression levels during oral squamous cell carcinoma development and progression [271]. This study demonstrated a statistically significant association between the decline in RAR β levels and the patient's response to isotretinoin [266, 271]. When non-exposed normal oral mucosa was compared to histologically normal mucosa from patients with oral lesions, p53 accumulation, loss of RAR β , and loss of the cell cycle regulator P21 were observed in the latter group [269, 271]. On multivariate analysis, the RAR β -/p21- phenotype was in fact associated with shorter disease-free survival [271]. It can be concluded that RAR β may contribute to the suppression of the premalignant phenotype whereas its loss may promote malignancy. The precise molecular mechanisms culminating in downregulation of loss of RAR β are not well-understood. Epigenetic silencing mediated by aberrant hypermethylation of CpG islands in the RAR β promoter was linked with the downregulation of RAR β . Decreased levels of RAR β were observed with Vitamin A deficiency and expression was re-stimulated by retinoic acid treatment, indicating that RAR β expression depends on the intracellular level of retinoids [266, 271].

6.2. Lotan and Chemoprevention

To understand the role of PPAR γ in cancer, it is useful to review the role of its heterodimer, the retinoic acid receptor (RAR). This is reviewed by Germain, et al. [273] RAR's are known to mediate both organismal and cellular influences of retinoid compounds. The term "retinoids" is generic and refers to compounds including both naturally dietary vitamin A (retinol) metabolites and synthetic analogs [274, 275]. Experimental and clinical research have demonstrated that retinoids regulate a broad variety of essential biological processes, including vertebrate embryonic morphogenesis, organogenesis, cell growth arrest, differentiation, apoptosis, and homeostasis; this also includes disorders related to these functions [273, 274, 276-278]. All-trans-retinoic acid (ATRA) is the most potent biologically active metabolite of vitamin A; it can both prevent and rescue the main defects due to vitamin A deficiency (VAD) in adult animals [278]. Preclinical studies performed in 1925 revealed that VAD correlated with squamous metaplasia development in rodents [279]. Based on this and subsequent studies, a strong argument for the use of retinoids in the treatment and prevention of cancer was predicted [280]. The most remarkable example of retinoid anti-cancer activity is evident in the treatment of acute promyelocytic leukemia (APL), which is a subtype of acute myelogenous leukemia. With the addition of ATRA to therapy, approximately 72% of patients with APL can be cured [281-283].

6.3. RA and Diseases, Treatments, and Chemoprevention

Several diseases have been associated with RARs, including cancer, skin disorders, and others. Retinoids are included in a number of chemoprevention and chemotherapeutic settings. The use of ATRA, 9CRA, and 13-cis-retinoic acid for dermatological uses including acne, psoriasis, and photoaging demonstrates the potential of retinoids in skin diseases [284-286].

Abnormalities of retinoid signaling have been linked to carcinoma. The most explicit connection of RAR to human cancer is evident in APL, whose cause is secondary to reciprocal chromosomal translocation between RAR-alpha and promyelocyte leukemia

protein (PML) human genes; this leads to the alteration of the signaling of both RAR-alpha and PML [281]. The fusion protein product PML-RARalpha displays increased binding affinity to the transcriptional corepressors NCoR and SMRT as compared with RAR-alpha. This induces the recruitment of HDAC complexes and silences RAR target genes. This results in arrest of myelopoiesis at the promyelocyte stage and arrests differentiation of APL cells. Notably, the use of supraphysiological doses of ATRA leads to remission in patients with APL, highlighting the possibility for use of retinoids as chemotherapeutic agents. Elevated concentrations of ATRA can induce postmaturation apoptosis by inducing tumor-selective death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a potential molecule in cancer treatment [287]. Unfortunately, some patients with APL will become resistant and experience a relapse, becoming resistant to ATRA. It is remarkable that ATRA-resistant patients can achieve remission with treatment using Am80, a powerful retinoid analogue [288-290].

Translocation of RARalpha has been observed with other genes, including the promyelocytic leukemia zinc finger (PLZF) gene product, which is insensitive to ATRA. With the PLZF-RARalpha fusion protein, the PLZF component is constitutively associated to the corepressor complexes independently of ATRA. This is the suspected reason for ATRA insensitivity.

A large body of evidence indicates that retinoids pharmacologically prevent the development of cancer in a variety of tissues. Retinoids are used as chemopreventative treatments in pre-neoplastic diseases, including oral leukoplakia, cervical dysplasia, and xeroderma pigmentosum [291-293]. Preclinical studies did not consistently result in clinical response for the treatment of solid tumors. Both experimental studies and analysis of the natural course of solid human tumorigenesis indicate that RAR-beta may act as a potential tumor suppressor. The expression of RAR-beta may be selectively lost in types of cancer, including non-small cell lung cancer, squamous cell carcinoma of the head and neck, and breast cancer [294-296]. The combination of restoration of RAR-beta expression and concomitant retinoic treatment was associated with a clinical response of

oral leukoplakia [297]. In addition, RAR-beta-1', an RAR-beta isoform which arises from an alternative splicing of RAR-beta-1, may function as a tumor suppressor gene in lung cells and whose biological functions may be distinct from those of previously known RAR-beta isoforms [298].

Several studies have specifically investigated retinoic acids and HNSCC. 13CRA was used in one randomized study as an adjuvant treatment for recurrence. This resulted in the unexpected observation of a reduction of the incidence of second primary tumors in the treatment group. The study population included 103 patients who had undergone either radiotherapy or surgery of stage I-IV tumors. They were randomized to receive 13CRA (50-100 mg/m² per day) vs placebo for one year. Results at an average of 32 months follow up demonstrated that second primary tumors had developed in 4% of the treatment group and 24% of the control group. Interestingly, the drug had no effect on the rate of recurrence or metastasis [154, 299]. At the 55 months follow up point, the rate of second primary tumor development was 7% in the treatment group and 33% in the control group [300].

Bolla et al., evaluated etretinate (50 mg/day for one month then 25 mg/day thereafter for a total of 24 months) vs placebo in 316 patients who had been treated for early-stage HNSCC; he followed these patients for 5 years. He identified no difference in survival, disease-free survival, and the incidence of cancers between the study groups [301].

Retinoic acid has been shown in some cases to promote rather than inhibit cell survival; this effect is likely due to the promiscuous nature of nuclear hormone receptors. In the case of RAR's, heterodimerization with other members of the thyroid hormone receptor-like family is common, one example of which is seen with the PPAR family [268]. As a result, RAR/PPAR heterodimers activate the expression of pro-survival factors such as portions of the PDK-1/Akt pathway or survivin [268, 302]. Retinoic acid transporters CRABP2 and FABP5 are crucial to the balance of channeling of retinoic acid to these various heterodimer pairs [268]. CRABP2 overexpression leads to preferential

RAR/RXR activation and therefore inhibition of tumorigenesis. Conversely, overexpression of FABP5 leads to RAR/PPAR stimulation and activates carcinogenic genetic programs [268]. Of note, both proteins have been demonstrated to have differential expression in metastatic and HPV-associated HNSCC [303, 304]. The magnitude of this importance of these heterodimerization directing protein systems in carcinogenesis remains to be defined and may explain the diverse results for retinoid-based clinical trials in HNSCC [266, 273, 305].

To summarize, a logical basis for using retinoids in cancer chemoprevention and therapy was based on the different cellular and animal models with additional support by epidemiological data and clinical trial outcomes [229, 268]. Chemoprevention trials that included patients with increased risk for developing HNSCC revealed that retinoids have the capacity to suppress precancerous lesions and inhibit the formation of second primary tumors in patients who had been pretreated for an early-stage cancer [266, 273, 292]. Other studies using iso-tretinoin and other retinoids failed to demonstrate any benefit in second primary tumor development, recurrence, or mortality of HNSCC or lung cancer [266, 273, 305]. Trials are being undertaken to resolve this conflicting data by recruiting targeted study populations and by using novel drugs. The complexity of the molecular regulation of retinoid receptor biology and nuclear hormones in general make this a challenging process.

6.4. Peroxisome-Proliferator-Activated Receptors

As is the case with a variety of solid tumors, enhanced expression of PPAR β and PPAR γ has been demonstrated in HNSCC [263, 306, 307]. This has also been demonstrated in pleomorphic adenomas and adenoid cystic carcinomas and not in corresponding normal tissue samples [263]. PPAR γ agonist binding can induce cell differentiation, growth arrest, and apoptosis of cancer cells [259, 263, 307]. Based on this, synthetic PPAR γ ligands were evaluated as anti-cancer and chemopreventive drugs in a wide range of cancers, including HNSCC [263, 307]. At this point, the precise role of PPAR γ in

carcinogenesis, as well as the additional effects of PPAR γ agonists, has not yet been fully defined [263, 306, 307]. One example of conflicting data is shown by OSCC. In this case, researchers demonstrated that PPAR γ was overexpressed. However, PPAR γ inhibitors and not ligands interfered with adhesion and metastasis in vitro [263, 307]. It is possible that these effects are accounted for by the fact that PPARs can modulate (either directly or indirectly) key cancer-related pathways, such as Wnt- and NF κ B-signaling, in addition to their regulators [306, 308]. It is also possible that growth inhibition effects of PPAR γ ligands in HNSCC are a result of cross-talk with other nuclear hormone receptors, such as RARs [263, 266, 307]. This possibility is substantiated by the finding that the ligand-induced effects were dependent on both PPAR γ levels as well as the type of agonist/antagonist, their concentrations, and tumor types [263, 306, 308]. It is also probable that the variable pro-cancer and anti-cancer tumor effects of PPAR γ ligands are mediated by affecting the microenvironment of tumor cells, as may occur by affecting cancer-associated endothelial cells or fibroblasts [309]. Importantly, endothelial cell proliferation and migration, as well as angiogenesis, have been shown to be affected by PPAR γ [310]. While the precise mechanism is uncertain, hypoxia-induced angiogenesis can be targeted by PPAR γ ligands in the setting of cancer treatment [311]. Angiogenesis is known to be a crucial component of tumor development as well as therapy resistance and metastasis. PPAR γ ligand modulation of angiogenesis may therefore play a role in some of the clinical benefits which have been observed.

Several studies have examined the role of PPAR γ agonism in aerodigestive tract cancers, including SCC. Yoshida, et al., utilized the rat 4-nitroquinolone model of oral carcinogenesis and demonstrated a 40% decrease in incidence of oral squamous carcinoma and decreased multiplicity in rats treated with pioglitazone [312]. Similar reductions of rat tongue carcinomas were observed in a separate investigation using a different PPAR γ agonist, troglitazone [313]. These preclinical findings were supported by a large case control study looking at at-risk populations. In this large Veterans Administration study, researchers found a >40% decreased incidence of HNSCC and >30% decreased incidence of lung cancer among Veterans who were taking

thiazolidinedione agents for at least one year [314]. When combining lung cancer data with the health-care registry data, a strong rationale for investigating PPAR γ in chemoprevention can be made.

Several clinical trials have explored the potential benefit of PPAR γ for HNSCC treatment, however the outcomes have been mixed. Some trials demonstrated a 40% partial response rate; others could not demonstrate significant benefit [315, 316]. Additional trials are ongoing and more will be necessary to establish the role of PPAR γ ligands in the treatment of HNSCC. What can be said is that PPAR γ ligands may have potential for a role in the treatment of HNSCC. It appears that PPARs are targeted indirectly by various novel treatment approaches in HNSCC. One example of this involves COX2 inhibitors; they appear to also affect PPAR γ and may be generating an autocrine loop [317, 318]. Clearly, more study will have to be undertaken to determine modes of action, concentrations, and PPAR receptor function to determine an optimal role of PPAR γ modulation in the context of HNSCC treatment.

6.5. Peroxisom Proliferator-activated Receptors

6.5.1. Summary of NHR and Cancer. Early experiments have demonstrated the ability of lipoxygenase inhibitors to dramatically downregulate cell growth and development in a variety of cell lines [9-11]. Cell lines have been shown to decrease thymidine uptake, decrease in proliferation, and increase in lipid uptake when treated with a variety of lipoxygenase inhibitors; these changes cannot be entirely explained by the inhibition of lipoxygenase alone [7, 14]. Because some of these agents have since been identified as PPAR agonists, the possibility was invoked that some of their anti-cancer effects could be attributed not directly to lipoxygenase inhibition, but rather to PPAR γ activation.

Because cancer largely represents a disorder of differentiation, the possibility of driving malignant cells into a terminally differentiated state has been considered. Differentiation therapy has been successfully used in the treatment of acute promyelocytic leukemia

exhibiting the t(15;17) translocation with all-*trans*-retinoic acid [32, 33], hairy cell leukemia, and childhood neuroblastoma. Receptor agonism with retinoids has shown mixed results in clinical therapies for solid tumors or chemoprevention [2, 35, 319].

PPAR γ was identified as a nuclear transcription factor in adipocytes in 1994 (36) the powerful ability of PPAR γ activators to direct adipocyte differentiation was demonstrated the same year when ectopic expression and activation of PPAR γ in fibroblast cells was shown to cause their adipose conversion [39].

The induction of differentiation and cell cycle withdrawal in liposarcoma cell lines using PPAR γ ligands was demonstrated by Tontonoz [174]; evidence of terminal differentiation in response to PPAR activation was demonstrated in liposarcoma patients treated with PPAR γ agonists [175]. PPAR γ based differentiation therapy has been investigated in epithelial tumors. In breast cancer cell lines, activation of PPAR γ has been demonstrated to attenuate cellular proliferation through inhibition of cell cycle progression and induction of apoptosis; cause extensive lipid accumulation, decrease growth rate and clonogenic capacity; and exhibit a chemopreventative effect in an animal model [176, 234, 241]. Activation of PPAR γ in lung cancer cell lines has demonstrated to induce differentiation and apoptosis [23, 31].

Because the process of carcinogenesis fundamentally represents an aberration of differentiation, and because PPAR γ has been identified as major transcription factor in the control of differentiation, it is logical to examine the role of PPAR γ in the suppression of the early stages of carcinogenesis. Two major studies support the role of a direct mutation of PPAR γ in carcinogenesis. In the first, tumors from 55 patients suffering from sporadic colon cancers were examined for mutations in PPAR γ ; four of the 55 tumors demonstrated mutations in PPAR γ genes [193]. The second study investigated the PPAR γ and thyroid follicular carcinomas, revealing that a translocation event frequently occurs in these tumors. A PAX8-PPAR γ 1 fusion oncoprotein is formed [192]. The

potential role for PPAR to modulate the early events in carcinogenesis has spawned considerable interest in this transcription factor as a target for chemoprevention [27].

Because previously unrecognized PPAR γ ligands have been shown to induce morphologic changes associated with differentiation in earlier experiments in our lab, as well as anti-cancer effects in other cancer models, we examined SCCHN cell lines and tumor specimens for the expression of PPAR γ . We investigated the effect of activation of this transcription factor on growth and differentiation. Our results indicate that PPAR γ is present in SCCHN cell lines and native tumor specimens; activation of cell lines with a variety of PPAR γ ligands of different chemical classes causes morphologic changes consistent with a lipogenic phenotype and differentiation, growth arrest, as well as up-regulation of the receptor itself. We conclude that PPAR γ ligands may represent a class of drugs which have value in the treatment of SCCHN.

7.0. MATERIAL AND METHODS

7.1. Cell Culture

Mycoplasma free CA-9-22 and NA aerodigestive cell lines [320] were cultured at 37 C, 5% CO₂ as adherent monolayer cultures in RPMI 1640 Media supplemented with 2mM Glutamine, 10% heat-inactivated FBS (Gibco/BRL), 50 u/ml Penicillin, and 50 ug/ml streptomycin. Log-phase cells were routinely subcultured weekly after trypsinization.

7.2. Preparation of Nuclear Extracts

Extracts from log-phase squamous cancer cell lines were prepared according to the methods of Dignan, utilizing some of the modifications of Lee as previously published [12]. All samples were assayed in triplicate. Standard curves were generated by least squares analysis, to a zero order function utilizing Spectra Software. Correlation coefficients (r^2) for the functions were greater than .95 in all experiments.

7.3. Aerodigestive Cancer Cell Line Western Blotting

Cells were grown in 75 cm² or 150 cm² flasks to 60-80% confluence and treated with respective agents in serum free media. Nuclear and cytosolic extracts were prepared as described. Twenty ug nuclear extract protein were added to equal volume of 2x sample buffer (1.0 ml Glycerol, 0.5 ml beta-mercaptoethanol, 3 ml 10% SDS, 1.25 ml 1.0 M Tris-HCl, pH 6.7, 1.5 mg bromophenol blue) and loaded into a 1.5mm 10% polyacrylamide gel and run at 20 mA for 2 hours at 4 C. Gels were removed and incubated in transfer solution (25mM Tris Base, 192mM Glycine, 20% Methanol) for five minutes, then transferred to nitrocellulose membrane using the a semi-dry transfer unit (Hoeffer). Blots were blocked in 3% milk blocking solution (1XTBS, 3% Milk, 0.05% Tween 20) for one hour at 4 C. Following TBS rinse (10mM Tris-HCl, pH 8.0; 150 mM NaCl), blots were incubated in primary antibody at 1:1000 (Santa Cruz sc-7273 mouse monoclonal anti-human PPAR γ in blocking solution overnight. Blots were briefly rinsed, then incubated secondary antibody at 1:2000 (Santa Cruz goat anti-mouse HRP). Following rinse, the blots were developed using chemiluminescence technique using Santa Cruz reagents per manufacturer protocol and exposed to film.

7.4. Electromobility Shift Assays

7.4.1. Probe Preparation. Double stranded DNA oligonucleotide probes for OCT-1 were obtained commercially (Promega ,Madison, WI). The OCT-1 transcription factor consensus sequence contained 5'-TGTCGAATGCAAATCACTAGAA-3'. The DR-1 probe and mutant was obtained commercially and contained the sequence: 5'-AGC TTC AGG TCA GAG GTG AGA GAG CT-3' (Santa Cruz Biotech, Inc, sc-2547), 5'-AGC TTC AGC ACA GAG CAC AGA GAG CT-3' (Santa Cruz Biotech, Inc, sc-2548), respectively. The published PPAR γ recognition sequence for the Cyp4A1 probe was additionally utilized. The Cyp4A1 probe was manufactured by GibcoBRL as a sense and anti-sense oligonucleotide, as well as a mutated sequence. The Cyp4A1 probe contained the published Cyp4A1 sequence , 5'-TGA AAC TAG GGT AAA GTT CA-3'. The mutated sequence for Cyp4A1 was 5'-TGA AAC TAG CAT AAA GCA CA-3' with 4 base pair substitutions in the direct repeat element of the Cyp4A1 gene. The

Cyp4A1DNA strands were annealed by heating in annealing buffer (0.1 mM Tris HCL, 1.0 M NaCl, 1x React2 (BRL), 300 pM oligonucleotide) to 60° C, then cooling to RT. Consensus oligonucleotide probes were labeled with T4 polynucleotide kinase (Promega) and gamma-32-ATP (6000 Ci/mmol, Amersham, Arlington Heights, IL).

7.4.2. EMSA Binding Reactions and Shift Assays. EMSA binding reactions consisted of 5ug of nuclear extract protein identically as previously published [12]. The gels were dried and imaged by phosphor imaging with a Packard Cyclone Phosphorimager utilizing MP imaging screens and analyzed by Optiquant software (Packard Technologies, Downers Grove, IL). All EMSA reactions were carried out on at least 3 separate nuclear extract preparations. For supershift experiments, 1 ug of antibody for RXR alpha (sc 553x) or PPAR γ (sc7273) (Santa Cruz Biochemical, Santa Cruz, CA) was added 12 hours prior to the binding reactions with the labeled probes and the EMSAs were carried out at 4°C. Densitometry analysis was performed using Optiquant software.

7.5. Transient Transfections/Luciferase Assays

All cell lines were transiently transfected as with a PPRE reporter plasmid (TK-PPREx3-LUC [PPRE x 3(5'-TCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGAC), three copies] kindly gifted by Dr. Ron Evans, Salk Institute, LaJolla, CA). An additional luciferase construct for the promoter of the PPAR γ responsive lipid metabolism gene aP2 was utilized for a second series of experiments (kindly gifted by Dr. David Bernlohr, University of Minnesota). Briefly, cells at 60-80% confluence were exposed to 2ug/ml TK-PPREx3-LUC reporter (or aP2) construct plasmid and co-transfected with .4 ug/ml beta-Gal containing DNA reporter. Lipofectamine at 10 ug/ml in Opti-MEM (both from GIBCO, Gaithersburg, MD) was utilized for transfection. After 4 hours, the media was washed free from the cells and complete media was used for 24 hours further incubation. Relative luciferase activity was assayed with a Dual-Light reporter gene assay system (Tropix, Bedford, MA) as per kit instructions. A Tropix model TR717 dual injection plate luminometer with Winglow software was utilized for the analyses. Luciferase was

scaled to B-gal as an internal standard. For PPAR γ antagonist experiments, cells were pre-incubated for 1 hour with T0070907 prior to incubation with activators. Three assays were performed in triplicate wells for a total of nine replicates per data point.

7.6. Cell Proliferation Assays

Log-phase NA and CA-9-22 cells were plated in tissue culture treated 96-well, flat-bottom plates (Falcon, Becton Dickinson Labware, Franklin, NJ) at 5000 cells/well in complete media overnight. The following morning, media was replaced with serum free media containing PPAR γ ligands or the vehicle alone (ETOH or DMSO) for 72 hours. Proliferation was determined by using the MTT reaction assay (Boehringer Mannheim, Indianapolis, IN) as per manufacture instructions reading the plates on a Tecan plate spectrophotometer (Tecan, Durham, NC) at 560 nM. For PPAR γ antagonist experiments, cells were pre-incubated for 1 hour with T0070907 prior to incubation with activators. Six replicates were used for each data point and experiments repeated twice.

7.7. Clonogenic Assays

CA-9-22 and NA cells were trypsinized during log phase growth and 400 cells per well in 5% media and allowed to attach overnight. The following morning the agents or the appropriate vehicle controls were added to the wells. After 10 days cell colonies representing cell numbers of 25 or more cells were counted after staining with crystal violet. The treatments were conducted in triplicate, experiments were repeated with similar results twice, and results are expressed as fractional number of colonies over control.

7.8. Oil Red O Studies

Cell lines were grown to 50-60% confluence in 12-well flat bottom plates; respective ligands were added in 2% media and incubated for 72 hours. Cells were then harvested by trypsinization, resuspended and fixed in CytoRich (Tripath Imaging, Inc, Burlington, N.C.). Liquid cytology slides were made according to the method of Maksem [320]. Oil

Red O solution (0.2% in isopropyl alcohol) was then applied for 15 minutes; cells were then rinsed with serial concentrations of isopropyl alcohol and digital photographs taken.

7.9. Genomics Analysis

Patient characteristics and biopsy samples from patients and volunteer control subjects were obtained in accord with guidelines set forth by the Institutional Review Board of the Human Subjects Protection Committee at the University of Minnesota. The University of Minnesota Cancer Center Tissue Procurement Facility obtained tumor samples from surgical resection specimens from patients undergoing surgery for SCCHN using standardized procedures. All samples were immediately placed on ice and, within 30 minutes of devascularization, frozen in liquid nitrogen following removal of portions needed for pathological diagnosis. Histological analyses were performed to ensure that each specimen contained greater than 50% tumor tissue and less than 10% necrotic debris and those samples not meeting these criteria were rejected. Healthy control subjects without a history of oral cancer, pre-malignant lesions or periodontal disease were recruited through the University of Minnesota School of Dentistry. Following administration of a local anesthetic, a 6 mm punch biopsy of tissue was obtained from the buccal mucosa in the region adjacent to the third molar. Tissue specimens were flash frozen in liquid nitrogen and stored until extraction of mRNA. On average these tissues contained 40% epithelial mucosa and 60% submucosal tissue.

7.10. Extraction of Total RNA and Probe Preparation

50 to 100 mg of tissue was submerged in 1 ml of Trizol reagent (Life Technologies, Gaithersburg, MD) and immediately homogenized using a rotor-stator homogenizer (Powergen 700, Fischer Scientific) under RNase free conditions. Total RNA was extracted from the samples using the Trizol extraction protocol following a one-minute spin at 12,000 x g to pellet particulate matter. Total RNA was precipitated by incubating with 0.5 ml of isopropyl alcohol for 10 minutes followed by centrifugation at 12,000g for 10 minutes at 4°C. The pellet was washed twice with 75% ethanol, dissolved in RNase free water and stored at -80°C until further use. Agarose gel electrophoresis was

performed on each sample prior to further analysis to confirm the presence of non-degraded RNA.

Five to 10 microg of total RNA was used to prepare biotinylated cRNAs for hybridization using the standard Affymetrix protocol (Expression Analysis Technical Manual, Affymetrix, Inc., 2000). Briefly, RNA was converted to first strand cDNA using a T7-linked oligo(dT) primer (Genset, La Jolla, California), followed by second strand synthesis (Invitrogen Corporation, Carlsbad, CA). The double stranded cDNA was then used as template for labeled in vitro transcription reactions using biotinylated ribonucleotides (Enzo, Farmingdale, New York). Fifteen ug of each labeled cRNA was hybridized to Affymetrix U133A GeneChips (Affymetrix, Santa Clara, California) using standard conditions in an Affymetrix fluidics station.

7.11. Analysis of Microarray Data

7.11.1. Pre-processing of Microarray Data. Scanned Affymetrix array data was uploaded into the GeneData Cobi 4.0 (www.genedata.com) database maintained by the Supercomputing Institute's Computational Genomics Laboratory at the University of Minnesota. Pre-processing of the Affymetrix arrays was carried out using GeneData Refiner 3.0 (www.genedata.com) software to correct for variations in hybridization intensity due to gradient effects, dust specks or scratches. Gene expression intensity for each array was scaled to an arbitrary value of 1500 intensity units to allow comparisons across all arrays. Expression intensity values for each gene were derived using Refiner by applying the Microarray Suite 5.0 algorithm.

7.11.2. Statistical Analysis. Genes differentially expressed between the 41 SCCHN and 13 normal oral mucosal biopsies were identified using a Satterthwaite t-test [321] to robustly estimate significance despite unequal variance among groups. Further analysis was performed as previously published [322].

7.12. Tumor Specimen Western Blotting

The protein layer from Trizol extracted tumors was used for the tumor immunoblotting experiments identically as per manufacturer instructions with 40 uG tumor protein loaded per lane. Specimens were obtained from central portions of the tumor and contained tumor cells and surrounding stroma but not other subcutaneous tissues including adipose tissue. Normal mucosal specimens were likewise collected without submucosal tissue contamination. Specimens were supplied without patient identifying data, but the tumors for the Western blot experiment were from a different series of patients than the microarray experiments.

7.13. Biochemicals

10xTBE (Biofluids, Rockville, MD), Electrophoresis grade acrylamide and bis-acrylamide, PMSF, DTT, NaCl (Sigma Chemical, St. Louis, MO) , Nonidet P40 (U.S. Biochemiocal Corp., Cleveland, OH). MgCl₂, KCl (Quality Biological Corp., Gaithersburg, MD), EDTA (Research Genetics, Huntsville, AL), 1M HEPES, Glycerol (Gibco/BRL), Ammonium Persulfate, TEMED, 10x TBE (Bio-Rad Laboratories, Richmond, CA). 15-deoxy-delta-12-14-PG-J2, PPAR γ antagonist T0070907 (Cayman Chemical Company, Ann Arbor, MI), Ciglitazone, ETYA (Biomole, Plymouth Meeting, PA).

7.14. Statistical Analysis

All analysis were conducted utilizing 2 sided Student T-tests with 95% confidence intervals calculated with a statistical analysis program (Statmate, Graph Pad Software, San Diego, CA). The overall analysis of the Affymetrix chip data has been previously published [39]. For the analysis of the PPAR gene expression data a 2 sided t-test with a correction for unequal variances was employed (Welch Correction). CA 9-22 and NA Cells were a generous gift from Dr. Toshio Kuroka (Tokyo, Japan), and were authenticated by short tandem repeat profiling in 2010 [323].

7.15. Apoptosis Assays

Apoptosis assays were conducted using PARP cleavage assay using Western antibody techniques as described above. Cells were grown to 50% confluence in serum-containing media, then treated for various times in serum-free conditions. Equal protein loading was carried out and experiments were repeated in triplicate. Caspase assays were performed in 96-well plates and analyzed with colorimetric assay.

8.0. RESULTS

8.1. PPAR γ Protein Expression and Induction Squamous Aerodigestive Cancer

NA and CA-9-22 oral cancer cell lines were treated with PPAR γ activators and Western and electromobility shift assays performed. We used 3 structurally diverse PPAR γ activators including eicosatetraenoic acid (ETYA) (a synthetic analog of arachidonic acid known to have anti-cancer properties), ciglitazone (a thiazolidinedione), and prostaglandin 15-deoxy-delta-12-14-PG-J2 (PG-J2). At baseline, small amounts of immunoreactive PPAR γ protein were present at the anticipated molecular weight in nuclear extracts from both cell lines and this was increased after 40 hours of ETYA, ciglitazone, and PG-J2 treatment (see Figure 1A). The 40 hour time point was chosen for these initial experiment to be consistent with original experiments on PPAR activation [18, 39]. These experiments were repeated three times with similar results.

Next, to confirm the Western finding, we incubated CA-9-22 cell nuclear extracts with 10 μ M ETYA for 40 hours and then performed EMSAs utilizing the published PPAR γ recognition sequence from the cyp 4A1 gene promoter [46]. Nuclear extracts from untreated CA 9-22 cells contained small amounts of protein as demonstrated in lane 1 of Figure 1B. This protein supershifted with antibody to PPAR γ and RXR α (lane 3 and 4), demonstrating that both binding partners for an active heterodimer exist in the nuclear

extracts of CA 9-22 squamous carcinoma cells. Ten μM ETYA treatment caused increased binding of proteins in nuclear extracts to the cyp 4A1 PPAR γ recognition sequence (lane 5). Supershift analysis with PPAR γ and RXR α antibodies demonstrated 3.3 fold increases in PPAR γ binding (Lane 7) compared to untreated CA 9-22 cells (Lane 3). Unlabeled competition experiments with 100-fold excess of the Cyp4A1 oligonucleotide are shown in lane 2 and 6. The experiments were repeated and Oct-1 was used as a housekeeping control for the experiments (not shown). These data demonstrate that oral cavity cancer cells have the capacity to upregulate nuclear PPAR γ nuclear protein in response 3 classes of PPAR γ activators (see Figure 1), and that the upregulated nuclear protein has the capacity to bind to a PPAR γ response element containing a PPAR γ /RXR α dimers after ETYA treatment (see Figure 1).

To analyze functional activity of the PPAR gene, functional analysis using the PPAR γ luciferase reporter gene experiments were conducted. Figure 2 demonstrates significant activation of the PPAR luciferase reporter after 24 hours treatment in CA-9-22 and NA cells with ETYA (see Figure 2A, B $p < 0.0001$). Next, PG-J2 treatment of both cell lines caused increases in the PPAR luciferase reporters ($P < 0.0001$). The maximal activation of the reporter genes occurred at a $5\mu\text{M}$ concentration of PG-J2 in both cell lines (see Figure 2C, D). Above $5\mu\text{M}$, under the reporter gene conditions, apoptosis/cytotoxicity was often evident by inverted phase light microscopy. Similarly, ciglitazone treatment of both cell lines caused increases in the PPAR reporters in both cell lines (see Figure 2E, F, $p < 0.0001$). The maximal activation of the reporter genes occurred at $5\text{-}10\mu\text{M}$ concentration in both cell lines. These data give functional support to the Western blots and EMSAs in Figure 1. Interestingly at $20\mu\text{M}$ of ciglitazone, cells developed an apoptotic appearance by light microscopy with floating cells present in significant numbers. Consequently, we found inconsistencies in the activations at concentrations of PG-J2 and ciglitazone above $5\mu\text{M}$. This was accompanied by 50% or greater decreases in the amount of βgal activation (data not shown), probably owing to apoptosis/cytotoxicity.

Figure 1

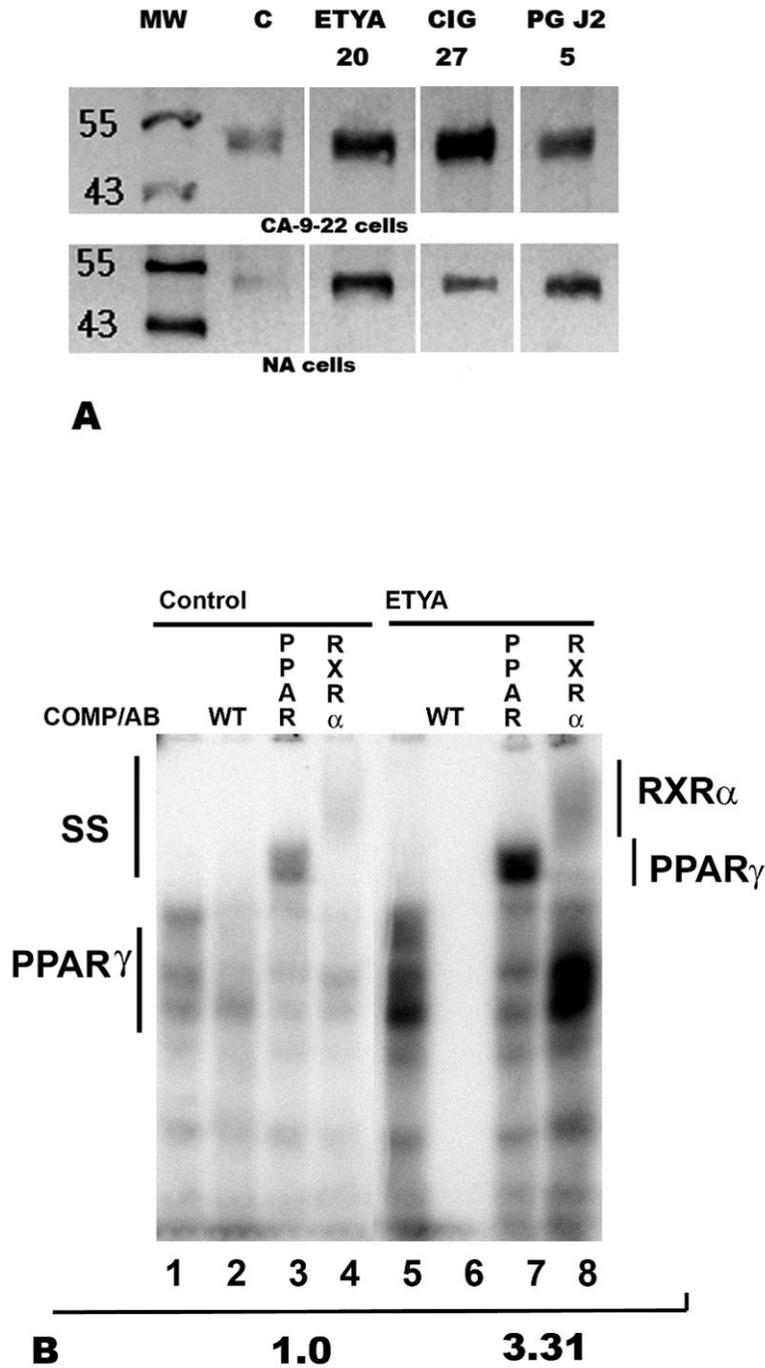


Figure 1. A Western blot analysis of nuclear extracts from cells treated for 40 hours with ETYA, Ciglitazone, and PG-J2. Concentrations are given in μM . Molecular weight markers (MW given in kD at the expected weight of PPAR γ). B: EMSA analysis of CA-9-22 oral cancer cells treated for 4 hours with ETYA. PPAR γ heterodimers are supershifted in the SS bands with both PPAR γ and RXR α antibodies. Densitometry analysis shows 3.31 fold increase in PPAR γ supershift (Lane 7 vs. Lane 3). WT = wild type competition of the bands. Lanes 1-4 control, Lanes 5-8 ETYA treatment.

Figure 2

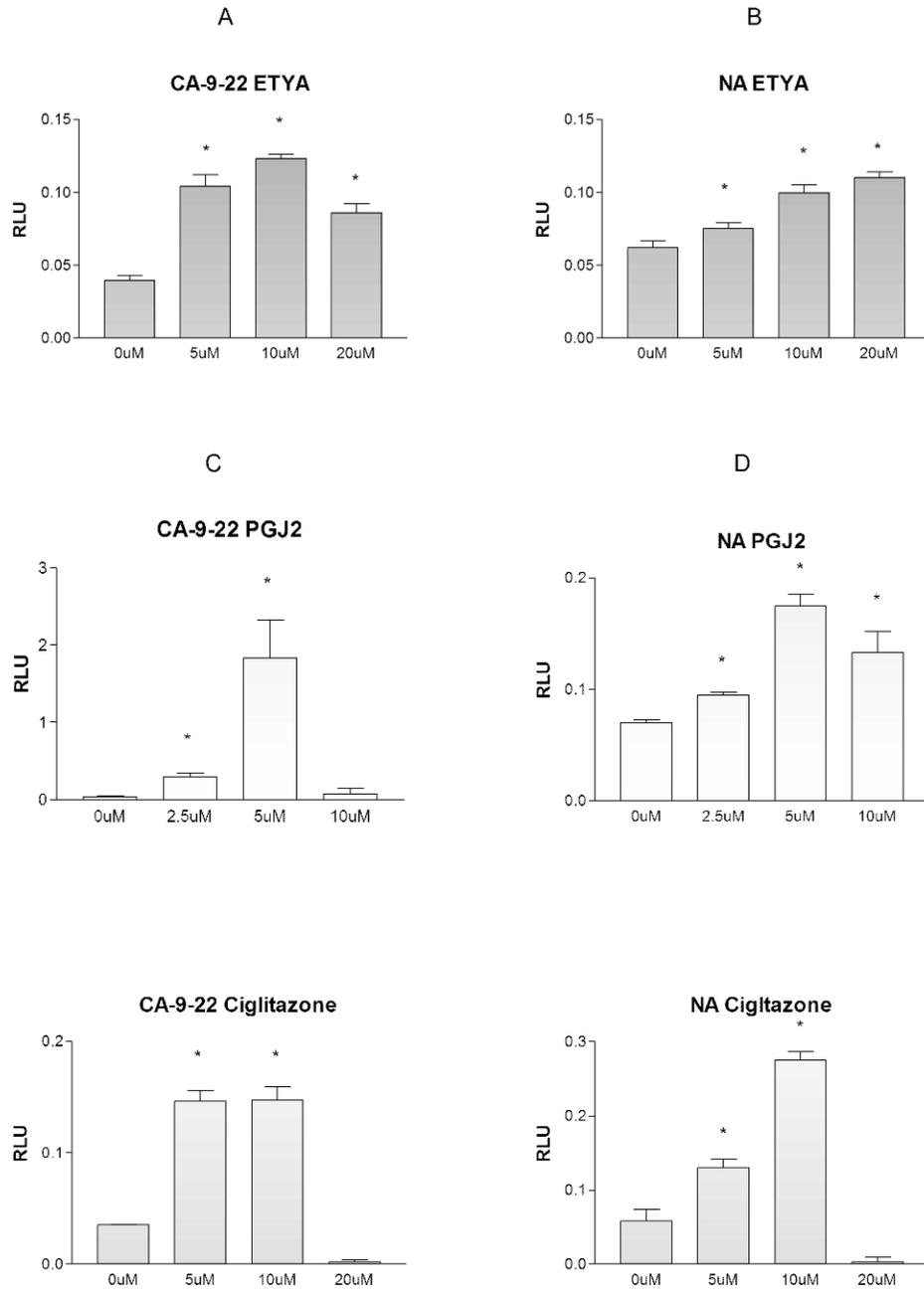


Figure 2. Reporter gene analysis of oral cancer cells treated with ETYA, PG-J2 , and Ciglitazone. Concentrations are given in μM . RLU are relative light units over the control for the treated cells. High concentrations of PG-J2 (10 μM) and ciglitazone (20 μM) were associated with cytotoxicity/apoptosis. “*” values signify a $p < 0.0001$. Experiments repeated 3 times. 9 replicates per data point depicted.

8.2. Ligands of PPAR γ Induce Growth Arrest and Decrease Clonogenicity of Squamous Aerodigestive Cancer Cells

Growth inhibition properties of PPAR γ inhibitors were investigated next with 3-day MTT assays. ETYA treatment of both cell lines resulted in decreased proliferation in a dose-dependent fashion at concentrations of 5 to 40 μ M ETYA (see Figure 3A and B). The EC 50 concentrations for the effects observed with ETYA are between 5 and 10 μ M for CA 9-22 cells and between 10 and 20 μ M for NA cells. Both cell lines demonstrated significant dose dependent decreases in cell proliferation with PG-J2 at 2.5 μ M (the reported EC 50 concentration) and above (see Figure 3 C and D) [28]. Ciglitazone caused similar dose-dependent effects with an IC 50 for of approximately 5 μ M for both cell lines. In sum, three structurally diverse PPAR γ activators decreased cell proliferation at similar concentrations to the effects shown in Figure 1- 2, demonstrating an associative link between PPAR γ activation and an anticancer property of the activators in oral cancer.

Next, diminished growth, a second anti-cancer property, was assessed in CA-9-22 cells after PPAR γ ligand treatment. ETYA at 5, 10, 20 and 40 μ M was effective in significantly reducing clonogenic growth of both cell lines when assessed at 10 days. (see Figure 4A and B). The IC 50 for the effect was approximately 20 μ M in both cell lines (EC 50 for PPAR activation by ETYA is 10 μ M)[5]. With PG-J2 there were significant dose dependent decreases in clonogenic growth after treatment with all concentrations of the agent tested (1,2,5,and 10 μ M). The IC-50 for clonogenic growth was between 1 and 2 μ M (reported EC 50 for PPAR γ activation with PG-J2 is 2 μ M). At 10 μ M, PG-J2 clonogenic growth was nearly abolished with greater than 95% inhibition of colony formation. With ciglitazone, the effects were less pronounced. Ciglitazone at 4.5 μ M had little effect on clonogenic growth, but concentrations of 9 and 18 μ M had significant effects, reducing growth by up to 40 % after treatment, with dose dependence. Each experiment was repeated with similar results. Taken together, these results demonstrate that 3 structurally diverse PPAR γ activators decrease two hallmark features of the neoplastic phenotype, proliferation and clonogenic growth.

To confirm the specificity of PPAR γ effects, antagonist studies were performed. After screening a variety of PPAR γ agonists, we utilized T0070907, a PPAR γ antagonist that covalently binds to Cys313 of PPAR γ inducing conformational changes blocking the recruitment of transcriptional cofactors to PPAR γ /RXR heterodimer. We preincubated CA-9-22 cells for one hour and then performed luciferase reporter gene assays as well as MTT assays. Reporter gene assays revealed significant reversal of PPAR γ reporter gene activation when T0070907 was co-incubated with all three PPAR γ activators. Additionally we wanted to investigate whether these results would translate to other observations we made. Therefore, we then performed cell proliferation assays with MTT. With ETYA and ciglitazone, significant reversal of inhibition of cell proliferation occurred with cell populations returning 20% with ETYA and 15-20% with ciglitazone. With PG-J2 and T0070907, however, this effect was absent (Figure 5). This likely reflects PPAR γ -independent antiproliferative effects on CA-9-22 cells. We next repeated the clonogenic assay experiments with T0070907 and CA-9-22 Cells. We observed that the antagonist itself had no effect on clonogenic potential of the cells. However, in the case of both ETYA and Ciglitazone (Figure 6A and C) T0070907 antagonized both ETYA and Ciglitazone. However, the results of PG-J2 demonstrated that only minimal insignificant changes in clonogenic growth after PG-J2 + T0070907 were observed after 5uM PG-J2. However, there was a significant change in clonogenic growth in the cells treated with 2.5 uM PG-J2 (35% of control colonies) versus PG-J2 and T0070907 (47% of control colonies, P=0.15). This is consistent with the MTT data that suggested that PPAR γ independent effects on cell growth were more likely to occur with PG- J2.

Figure 3

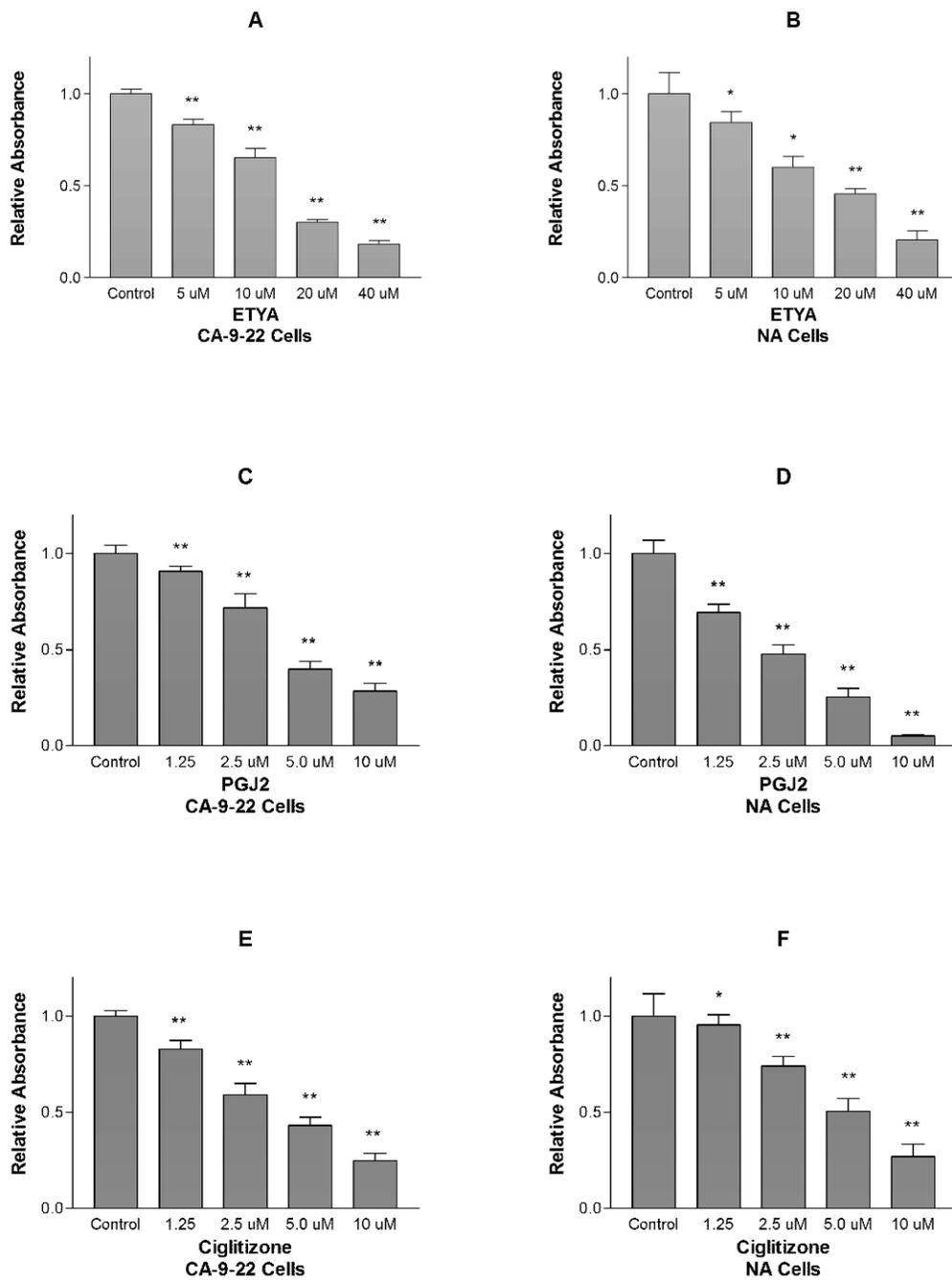


Figure 3. Growth inhibition in oral cancer cell lines with PPAR α activators. Dose dependent inhibition was noted with all 3 inhibitors. “*” signifies p<0.05, “” signifies p<0.0001. Six replicates per group, experiments repeated 3 times.**

Figure 4

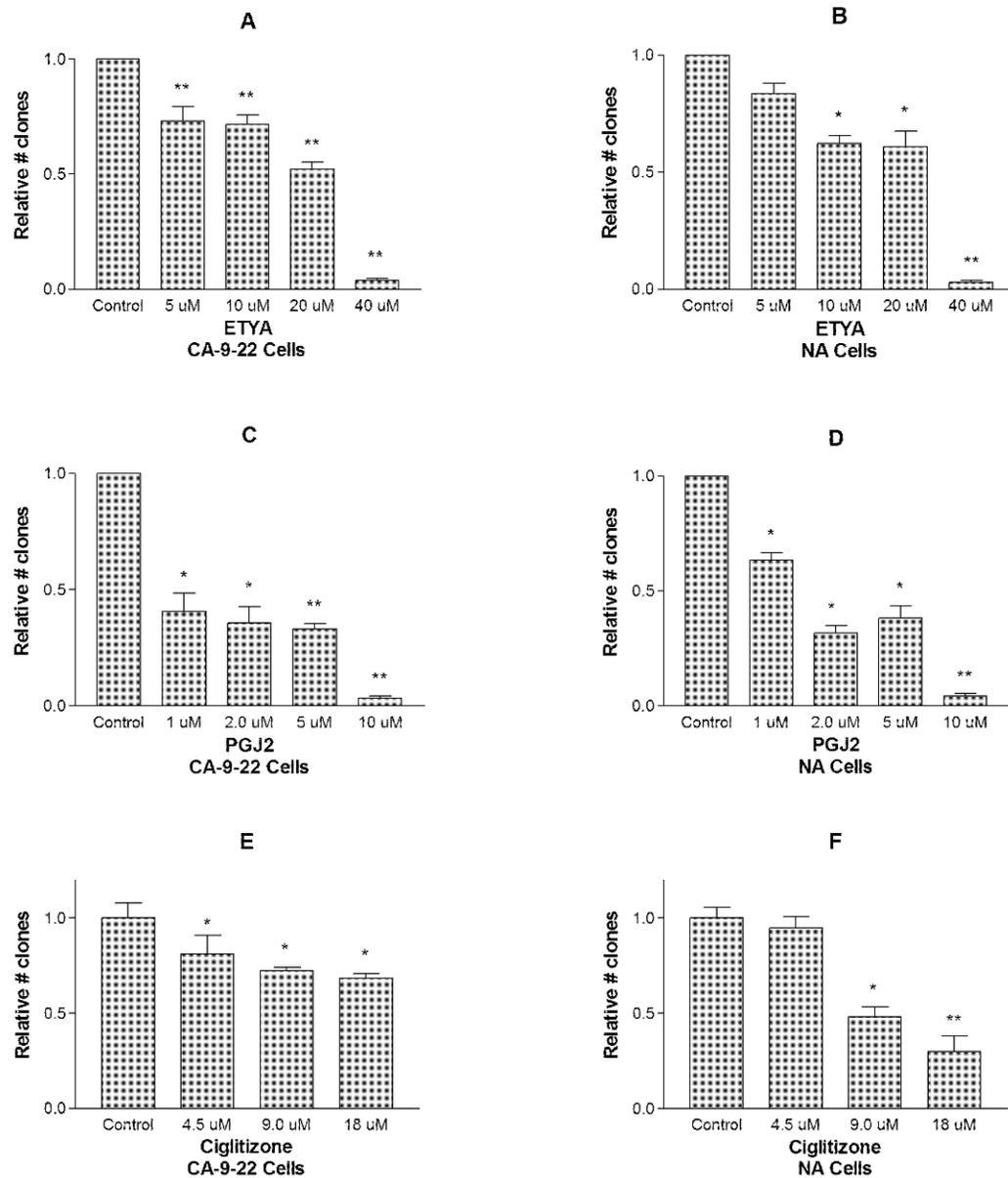


Figure 4. Clonogenic assays of oral cancer cell lines with PPAR α activators. Inhibition was noted in all three inhibitors. “*” signifies $p < 0.05$, “” signifies $p < 0.01$.**

Figure 5

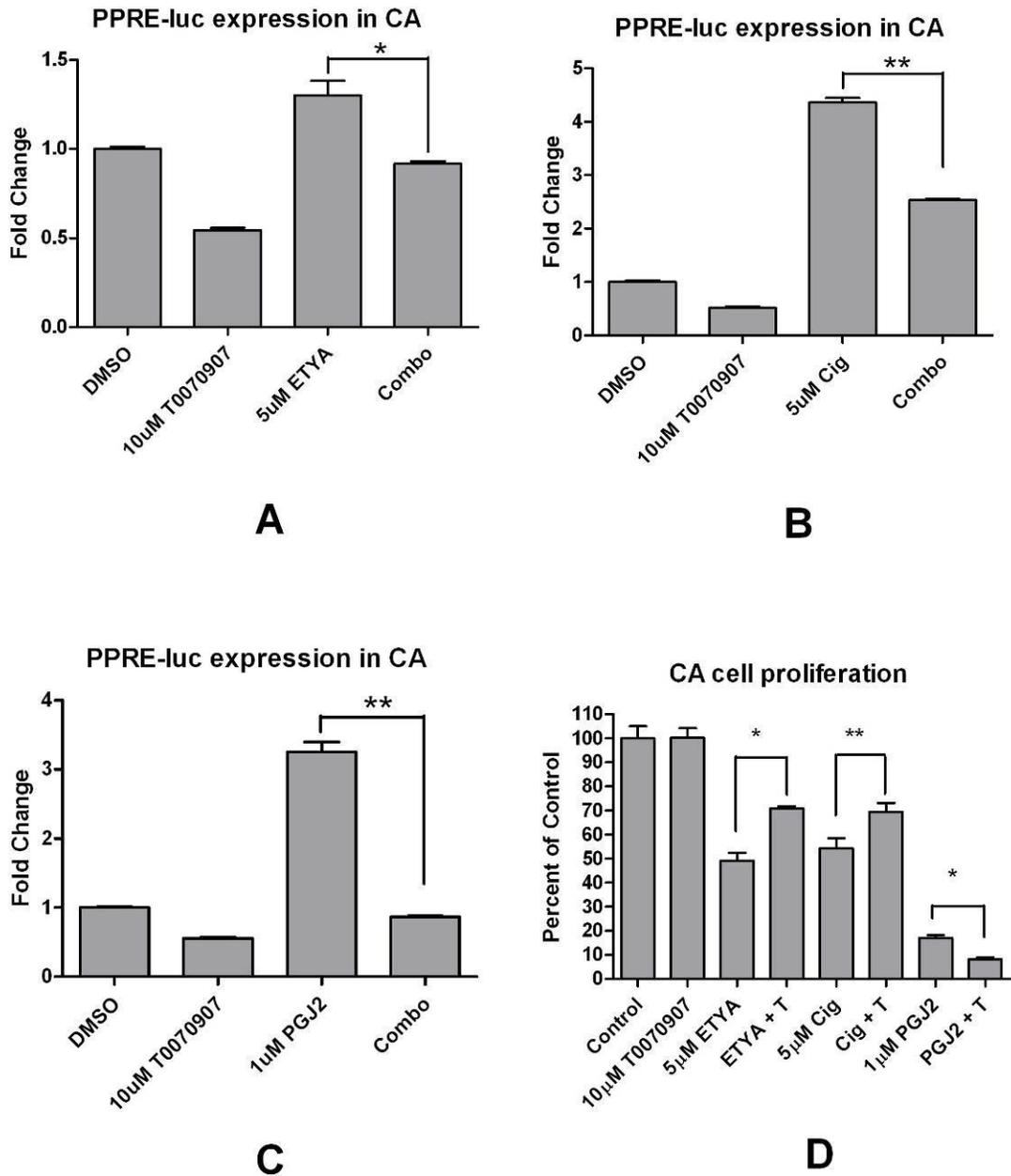


Figure 5. Treatment of HNSCC cells with PPAR γ antagonist abrogates effect of PPAR γ ligand treatment. A, B, C) Cells were transiently co-transfected with PPREx3-luciferase reporter gene and β -galactosidase reporter gene to account for transfection efficiency. After 24h treatment with PPAR γ ligands and antagonist, lysates were assayed for luciferase activity. Reporter activity is expressed as fold over control. Panels are representative of two similar experiments. 9 replicates per data point are utilized. *: $P \leq 0.003$, **: $P < 0.0001$. D) Cells were assayed for changes in cell proliferation via MTT assay after 72 hour treatment with PPAR γ ligands and antagonist. Cell proliferation is expressed as % of control and is representative of two similar experiments. 7 replicates per data point were utilized. *: $P < 0.0001$, **: $P = 0.0177$

Figure 6

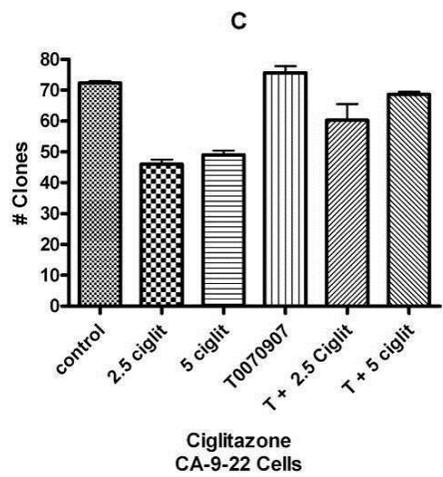
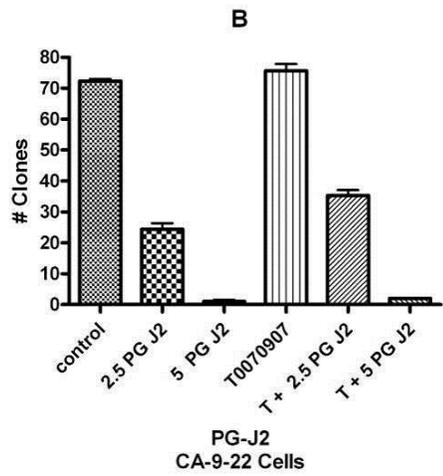
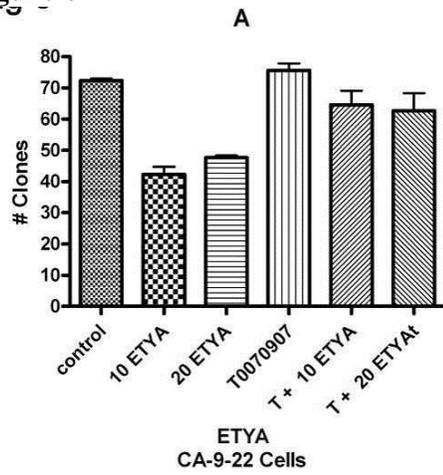


Figure 6: Clonogenic assays with T0070907 PPAR γ receptor inhibition in CA-9-22 cells. Cells were pre-incubated with T0070907 for one hour, then treated with respective agonists. Reversal of

clonogenic expansion was seen for ETYA and ciglitazone (p-value <0.03), but was much less pronounced for 10uM PG-J2 (p-value = 0.21). Error bars represent 95% confidence intervals.

8.3. Ligands of PPAR γ Induce Lipid Accumulation in Squamous Carcinoma Cells

The effect of PPAR γ ligands on cell morphology was assessed by light microscopy using oil-red-o staining (Figure 7). Cell lines treated with PPAR γ ligands demonstrated a morphologic change characterized by the acquisition of cytoplasmic lipid droplets. Cell pseudopod formation was observed in many cells in the plates, which constitutes differentiation for some squamous epithelia [324]. At higher doses, cell rounding and decreased cell density was typically observed, consistent with cell death and inhibition of proliferation, respectively. Even in these cells, lipid droplets are observed. Decreased cell density was a consistent finding across cell lines and all PPAR γ ligands. Vehicle-only control cells grew to high densities. These experiments were repeated several times with similar results.

To test the hypothesis that PPAR γ activators may cause transdifferentiation into an adipose phenotype, cells were tested for their ability to activate an adipose differentiation-specific gene aP2 (aka FABP 4). In both CA-9-22 cells and NA cells, ETYA at 10 and 20 uM activated the aP2 luciferase reporter gene, with significant activation occurring with as little as 5 uM ETYA (see Figure 8 A, D). Ciglitazone treatment demonstrated significant dose dependent increases in aP2 reporter gene activation at 4.5, 9, and 18 uM concentrations of ciglitazone for CA-9-22 cells (see Figure 8 B, E). Similar levels of activation were observed with PG-J2 treatment. Next RT PCR was conducted with PG-J2 and these experiments demonstrated increased FABP and adipsin expression after PG-J2 treatment, without increases in PPAR γ expression. In aggregate, these data demonstrate increased activation and expression of adipose differentiation-specific genes after PPAR γ ligand treatment, without the stimulation of new PPAR γ expression, confirming other preclinical data in the manuscript.

Figure 7

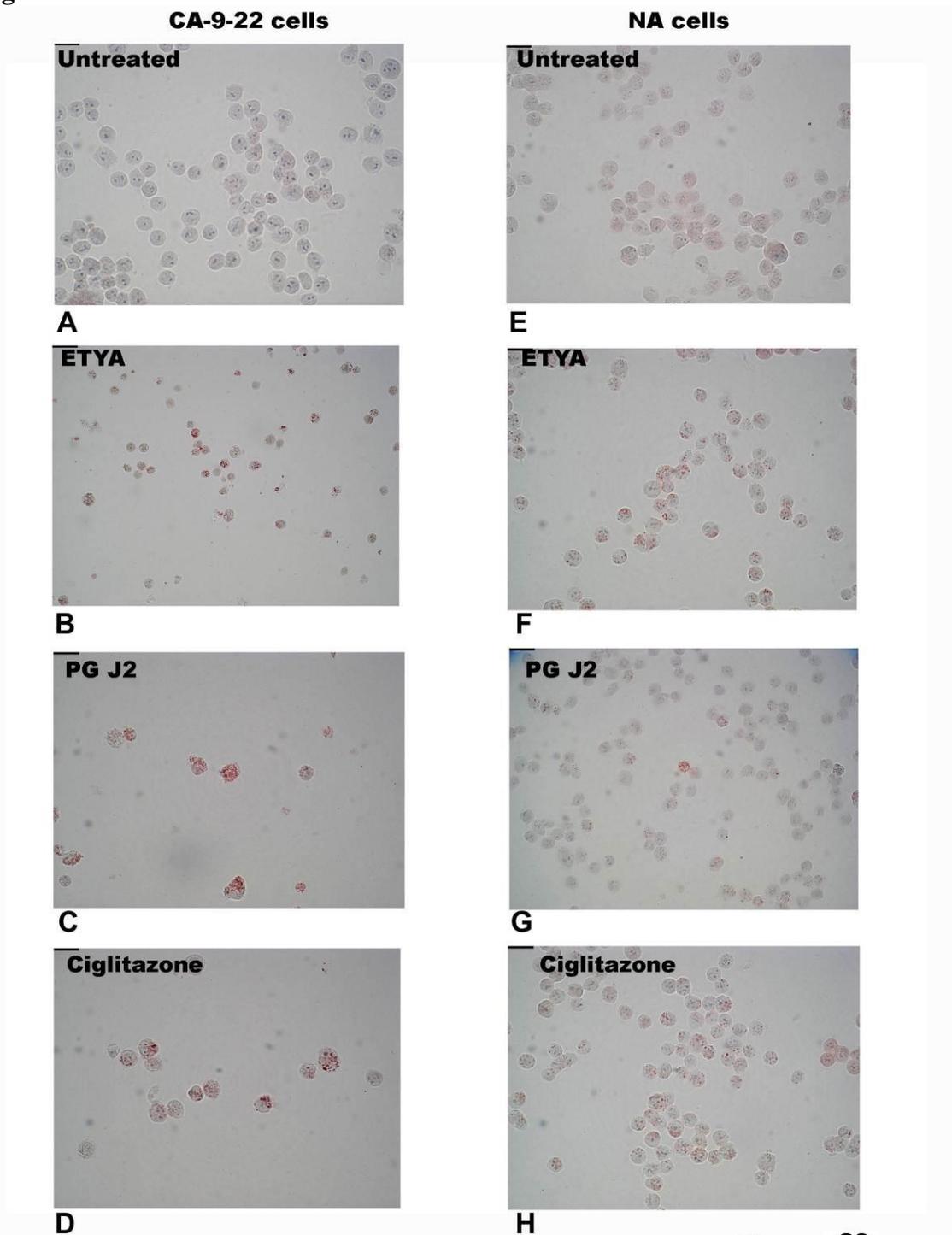


Figure 7. Oil red O staining for lipid accumulation. Cells were treated with the inhibitors for the specified time and cells were stained for Oil red O for lipid accumulation. Representative sections shown (100x magnification). Upper left bar represents 50 uM marker.

Figure 8

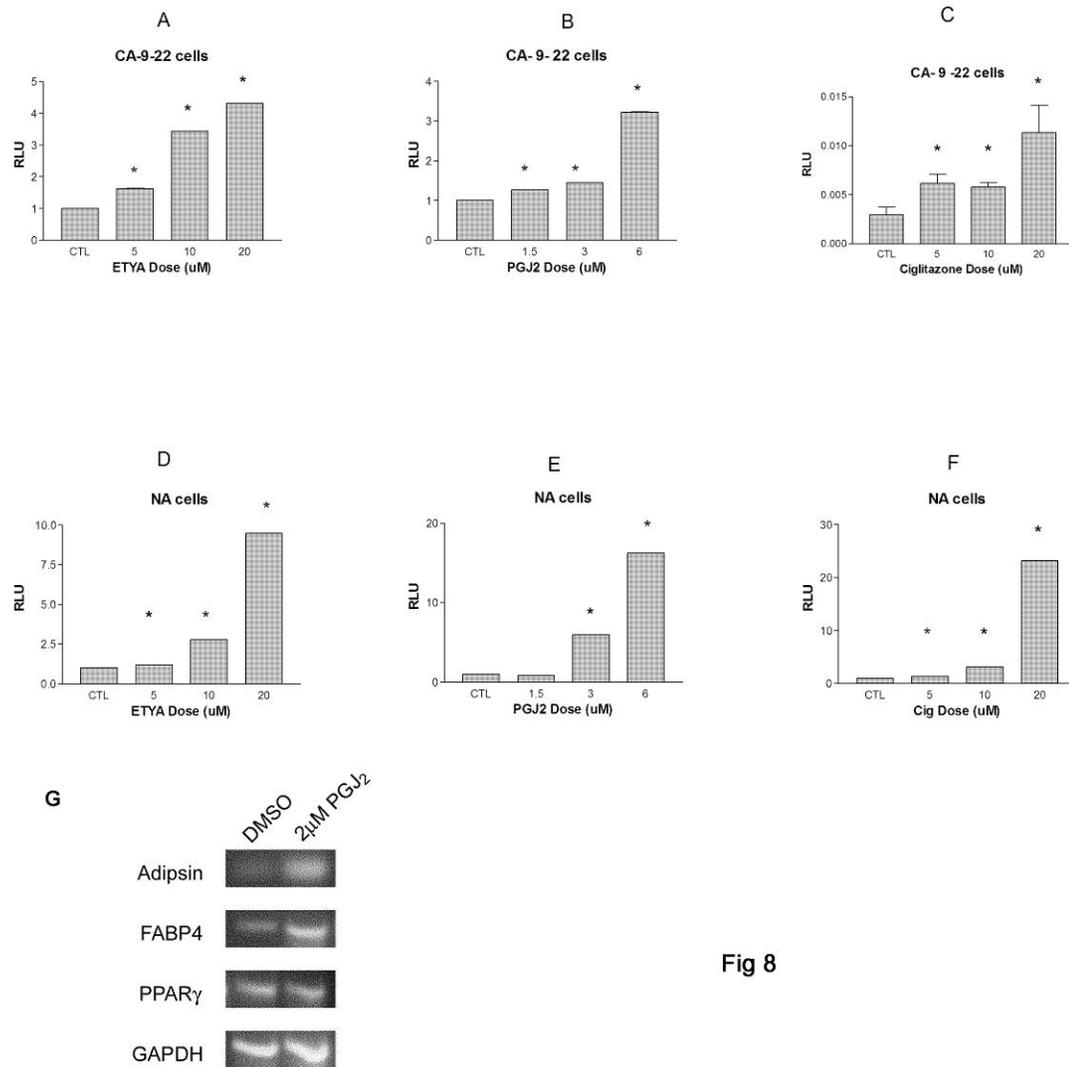


Fig 8

Figure 8. Lipid associated gene activation after PPAR γ activation. Ap2 (FABP4) Luciferase reporter gene activation after PPAR γ activation is depicted in A-F. Cells were treated for 24 hours and analyzed. There were 9 replicates per group. Error bars are enclosed within the lines of the Figure at a 95% CI. All “*” values are significant at p<0.0001. Representative of 2 experiments. Figure 7G demonstrates RTPCR of upregulation of FABP 4 (Ap2) and Adipsin after PG-J2 treatment for 24 hours.

8.4. PPAR γ Expression in Head and Neck Tumors

In addition to the identification of PPAR γ in NA and CA-9-22 cells, specimens from our head and neck cancer tumor bank/data base were interrogated for their level of PPAR γ expression using microarray technology. Thirty-four of 54 tumor specimens expressed

PPAR γ at detectable levels (63%) whereas 100% (13/13) normal oral cavity specimens expressed PPAR γ (see Figure 9A, B). As a group, the normal oral cavity specimens had a higher level of expression of PPAR γ (342.6 ± 87.22) than the tumors (180.7 ± 45.26) ($p = 0.0023$). In addition, PPAR α and δ were also interrogated from the array. There were no significant differences in PPAR α ($p = 0.5875$) expression in the tumors versus the control specimens (see Figure 9 B, E), but PPAR δ expression was significantly diminished in the tumors (833.1 ± 38.11) compared to the controls (1163 ± 89.9) ($p = 0.0038$) (see Figure 9C, F).

Western analysis also revealed PPAR γ positive immunoreactivity in 6 of 9 head and neck cancer specimens at the expected molecular weight of PPAR γ (see Figure 9G). Three of the specimens exhibited 2 separate bands at molecular weights reported for PPAR γ 1 and 2 isoforms suggesting that both isotypes are present in head and neck tumors [325]. The percent of tumors that expressed PPAR γ on gene bank interrogation and Western analysis was similar (66 versus 63%).

Figure 9

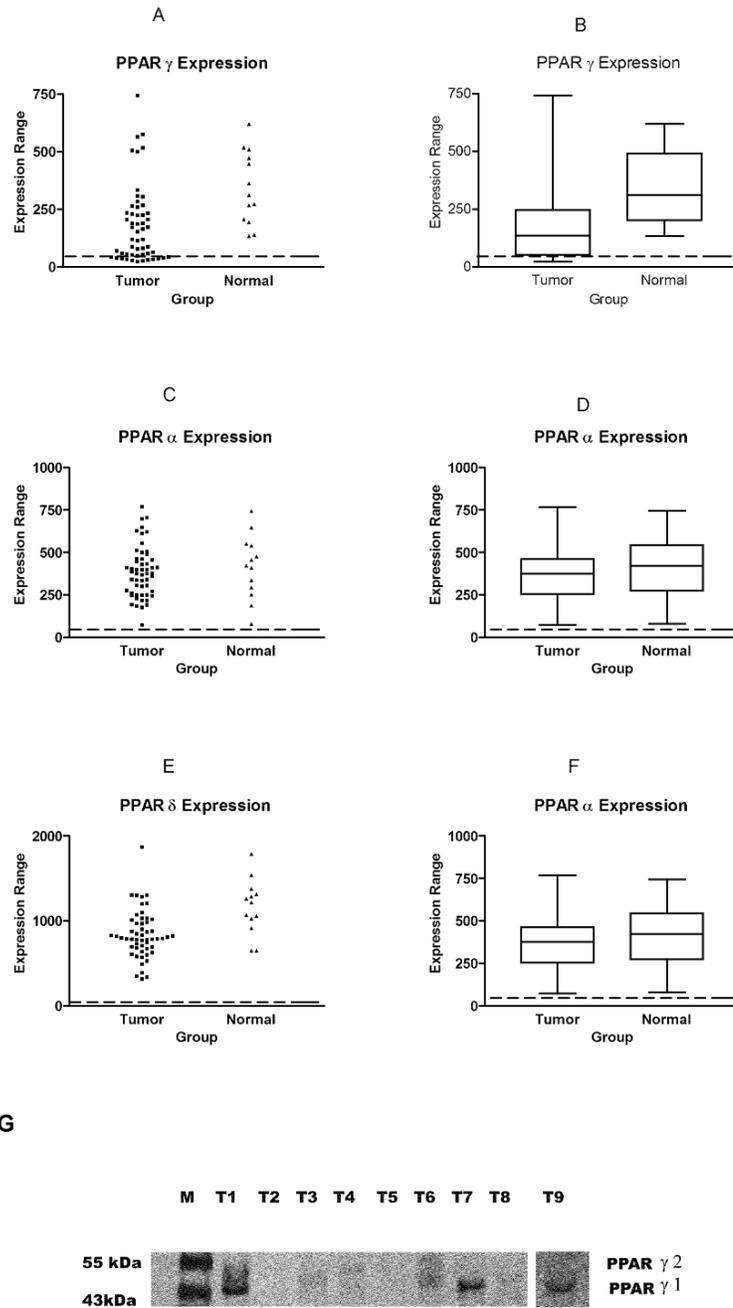


Figure 9. PPAR receptor expression in head and neck cancer specimens. Figure 8 A-F represents scatter and box-whisker diagrams of PPAR γ , α , and δ expression in head and neck cancer specimens and normals from our genomic database. The horizontal dotted lines represent cutoff for baseline expression of PPAR subsets. PPAR γ and δ were significantly downregulated in the specimens as described in the text. Figure 8G represents Western blot analysis of 9 tumors for PPAR γ . Six of the 9 tumors were scored + for expression with equal protein loading of all specimens.

8.5. Apoptosis is induced by PPAR γ activators

Apoptosis was assessed using PARP cleavage through Western analysis of cell homogenate and through caspase assays. PARP cleavage was assessed at twelve hours using PGJ2, ETYA, and CTZ. We observed cleavage for PGJ2 and ETYA but not CTZ. We elected to investigate further with performing caspase 3/7 assays. Under the conditions of the assay, we had to get to 9 μ M of ciglitazone to demonstrate statistically significant caspase induction. (Figure 10). Both cell lines demonstrated cytotoxic effects using high-concentration combined agent treatment.

Figure 10

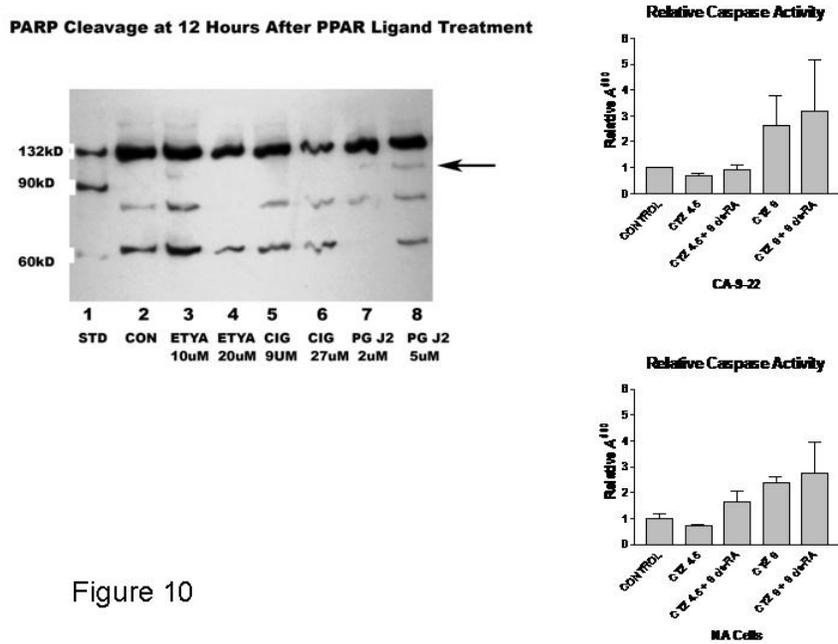


Figure 10

9.0. DISCUSSION

Since upper aerodigestive malignancies have not experienced substantial improvements in cure rates over years, and since no effective interventions for primary or secondary

chemoprevention have long-term safe efficacy, there is a vital and broad need to investigate new molecular targets for therapeutic interventions. Recent reports have demonstrated that a variety of PPAR γ activators demonstrate anticancer properties in solid tumor carcinogenesis including 4NQO tongue carcinogenesis in rats [326]. Further, a large analysis of over 85,000 diabetic patients on thiazolidinediones revealed 33% decreases in lung cancer rates in that population compared to control patients [326].

In the current study, we identified PPAR γ receptors in human squamous aerodigestive cancer cell lines and tumors. Additionally, we demonstrated that several classes of PPAR γ activators, *in vitro*, have the capacity to decrease cellular proliferation, clonogenicity and promote a differentiated phenotype, which appears to be some form of adipocytic transdifferentiation. This preclinical evidence, in aggregate, provides the rationale to pursue the further investigation of PPAR γ activation as a treatment strategy for upper aerodigestive malignancy or premalignancy.

While PPAR γ has a well-established role in differentiation in the adipocyte, its potential role in the differentiation of squamous epithelium or upper aerodigestive squamous malignancy is not well investigated. It is possible that this is related to metabolic demands intrinsic to cancer growth. It is also possible that some anticancer effects are PPAR γ -independent. Thiazolidinediones have been shown to have PPAR γ -independent effects through a variety of mechanisms [327-331].

Early experiments examining the effect of ETYA on lymphoma, glioma, prostate, and head and neck cancer cell lines (under the premise that ETYA was an eicosanoid synthesis inhibitor) revealed the unexplained finding of lipid uptake in several of the cell lines, as well as growth inhibition [18, 19, 39]. When ETYA was found to be a PPAR activator at similar concentrations used for previously published experiments, we investigated the probability of PPAR-directed differentiation event as an explanation for lipid uptake observed in the other studies. ETYA has the capacity to activate PPAR α and PPAR γ [19], and ETYA treatment of 3T3-L1 pre-adipocytes stimulates terminal

differentiation, presumably through its PPAR γ mediated properties [5]. By utilizing ciglitazone and PG-J2 treatment to support the ETYA observations we were able to demonstrate that three distinct classes of PPAR γ activators promote upregulation of a partial adipogenic phenotype in these squamous cells. These results are strengthened by the PPAR γ antagonist experiments with T0070907. In these experiments we observed that decreases in PPAR γ reporter gene activation specifically antagonized by T0070907 were accompanied by decreased efficacy of PPAR γ agonists in similarly treated cells on proliferation assays. Presently, we are testing this hypothesis clinically in leukoplakia patients being treated with the FDA approved thiazolidinedione pioglitazone (NCI contract N-01-15000). In our experiments, however, we observed a clear shift in SCCHN phenotype, involving the formation of cytoplasmic lipid droplets. This event requires an organized series of cellular events consistent with adipocyte differentiation. In non-small cell lung carcinoma (NSCLC), similar PPAR γ ligands have been shown to induce upregulation of general markers of differentiation, including gelsolin, Mad, and P21. However, no lipid specific markers or oil red o staining was observed under the experimental conditions in this study [31]. The presence of similarly altered phenotype of the SCCHN cells, in addition with the upregulation of the adipose differentiation markers, suggests that several PPAR γ activators cause this change. Other data that support PPAR γ activation as cause for these events included 1) upregulation of 2 PPAR γ reporter genes, 2) nuclear PPAR γ protein upregulation on Western blot analysis, and 3) upregulation of PPAR γ / RXR α heterodimers on EMSA using the PPAR γ recognition sequence CYP4A1. Apoptosis studies indicate the presence of apoptosis markers in both inhibitors and with each cell line. This effect was potentiated by co-incubation with RXR activators.

Generally, differentiation is accompanied by decreased proliferation. The decrease in proliferation and clonogenicity we observed, in the context of lipid accumulation after FABP 4 upregulation, is consistent with a possible transdifferentiation phenomenon occurring in both squamous cancer cell lines tested. In pre-adipocyte cell culture models, the first step of adipocyte differentiation involves cell cycle and cell proliferation arrest.

More importantly, from a cancer treatment or cancer prevention perspective, proliferation and clonogenicity arrest are important prerequisites for therapeutic compounds.

Additionally, the evidence that differentiation phenomena are occurring in the cell lines adds an interesting dimension to the potential clinical translation of these findings. In particular, early phase clinical trials might include an intermediate endpoint biomarker panel related to squamous or adipocyte lineage differentiation in addition to more typical surrogate endpoints like proliferation markers or targeted endpoints (e.g. PPAR γ).

In conclusion, SCCHN contains PPAR γ protein which can be functionally activated with three structurally unrelated ligands for PPAR γ . The functional activation correlates with increased levels of PPAR γ protein in the nucleus, and EMSA experiments demonstrate that PPAR γ and RXR α heterodimers are found in nuclear protein extracts that bind to 2 separate PPREs. Reports using breast cancer, lung cancer, colon cancer cell lines, in combination with data presented here provide a strong rationale for targeting PPAR γ as a therapeutic modality in the treatment of common epithelial malignancies or their precursors.

10.0. NEGATIVE STUDIES

10.1. Western Blots

Initial efforts at Western blotting were unsuccessful. The initial basis for lack of success was primarily related to poor technique. While I had performed some Western blotting prior to medical school, he had been a number of years and the materials I was using were different. In addition, we did not have access for developing the blots; initially we were transporting the exposed films to the radiology Department at the University Minnesota Hospital system for development.

In Figure 10, the result was dismal. Even the molecular weight marker can be seen to be unformed. While some bands are evident, no meaningful information is derived from this study. In a later trial using different secondary antibodies, the molecular weight

marker seems to be evident, though sloppy technique has resulted in a poor development. This appears to be likely secondary to transfer of the gel membranes (see Figure 11). Challenges of length of exposure of the films were also a challenge; this was complicated by the need to perform gel exposures in a dark room. We do not have a red light and this was frequently done simply by feel. The results are apparent here in Figure 12 with clear overexposure. Later, much cleaner gels were produced by the combination of better technique, alteration of exposure techniques, including digital camera exposure, and attention to detail (see Figure 13).

Figure 10

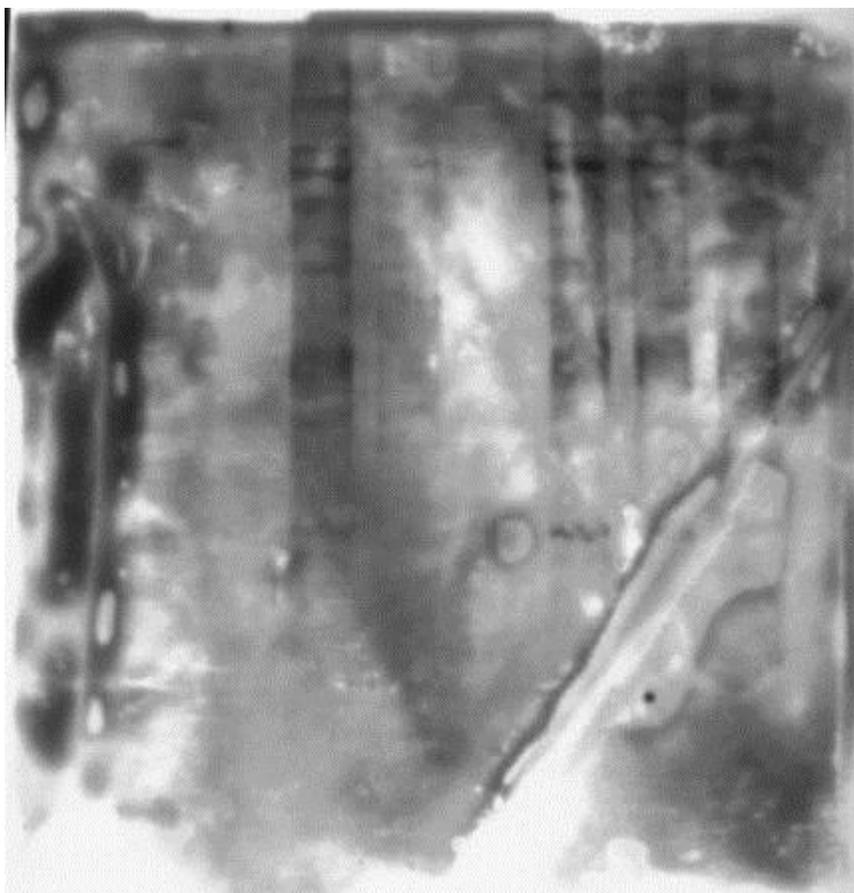


Figure 10

Figure 11

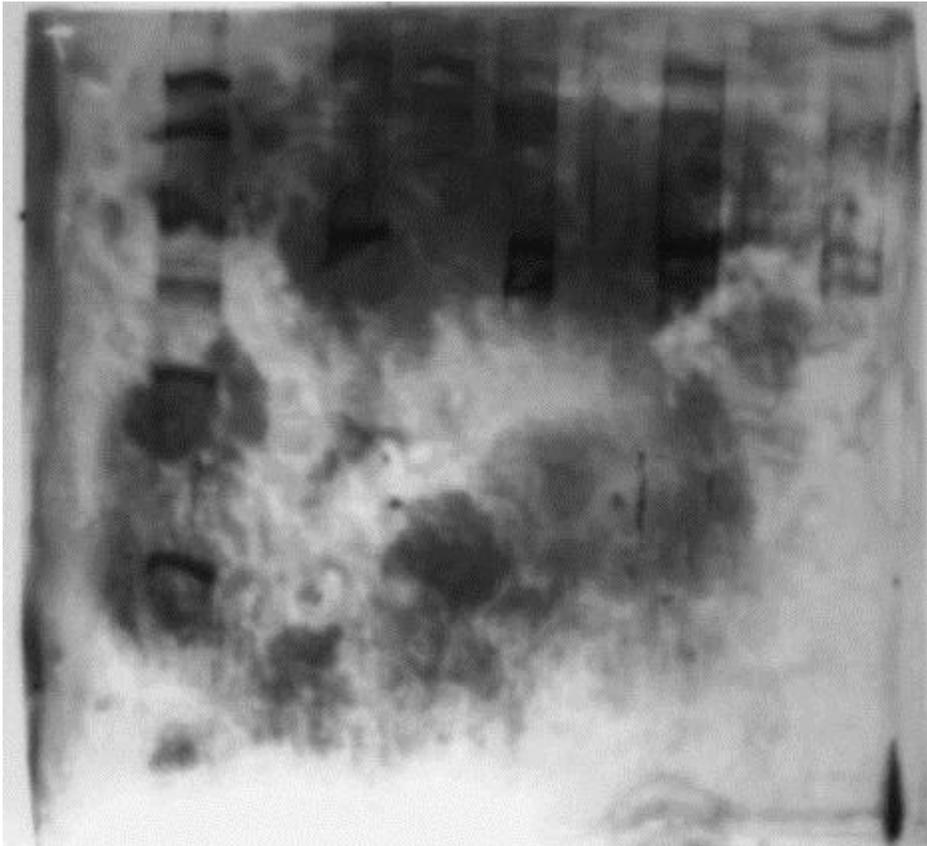


Figure 11

Figure 12

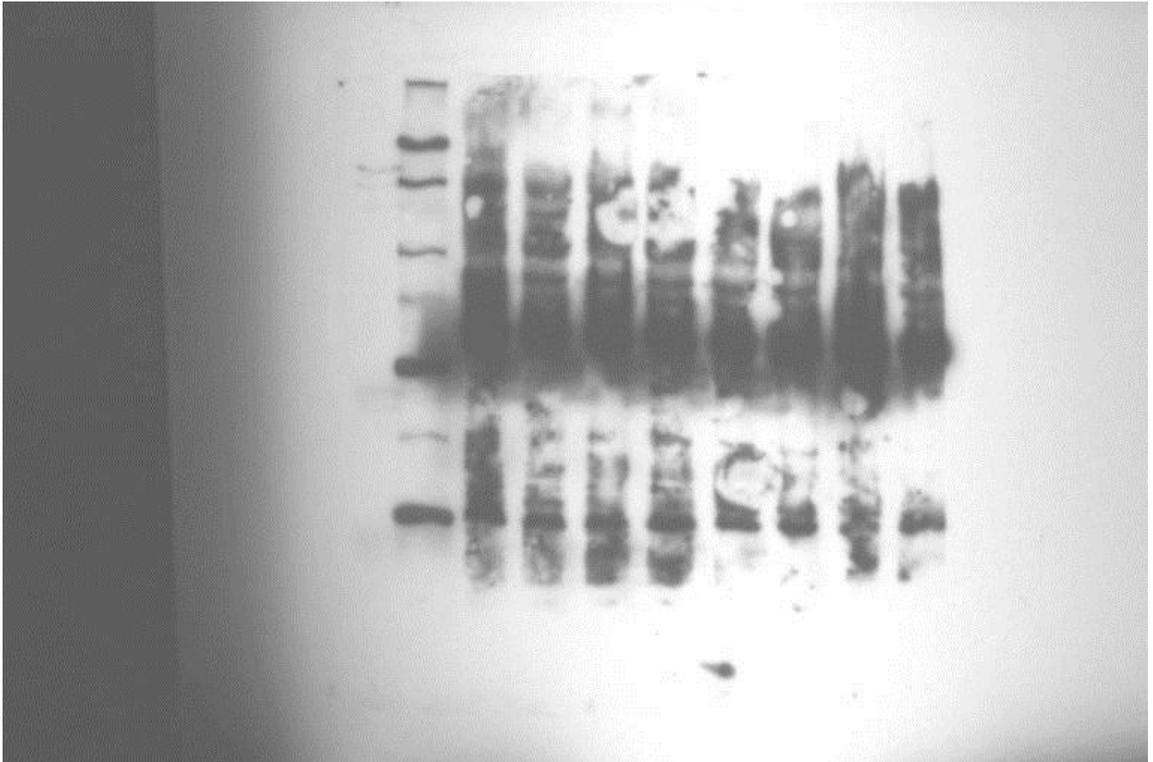


Figure 12

Figure 13

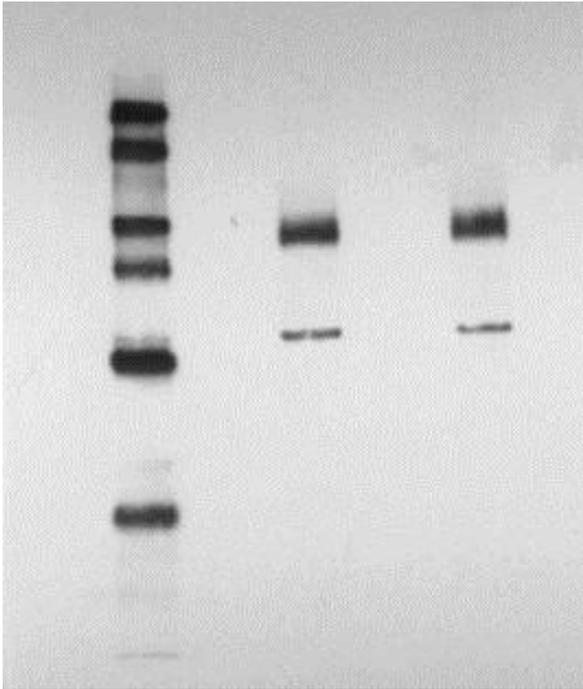


Figure 13

10.2. ORO Studies

Initial Oil-Red-O studies were also difficult. The main challenges were related to both preparation of cells using with or without serum. In addition, the variable of length of intubation was difficult to establish. Finally, the staining of the cells with oil red O. with complex. This required a variable length of staining followed by ethanol rinsing. Without care, it was possible to over rinse and remove some of the stain (see Figure 14). Incomplete rinsing resulted in over -staining of the cells (see Figure 15). Finally, capturing the image of the stain presented a problem with immature digital capture technology (see Figure 16). In addition, it was very difficult to maintain a controlled which would not go to confluence, yet allow enough time for the treatment cells across the range of concentrations were utilized. In this figure, we can see that the control cells

have grown to confluence (see Figure 17). A number of very poor experiments were produced.

Figure 14



Figure 14

Figure 15

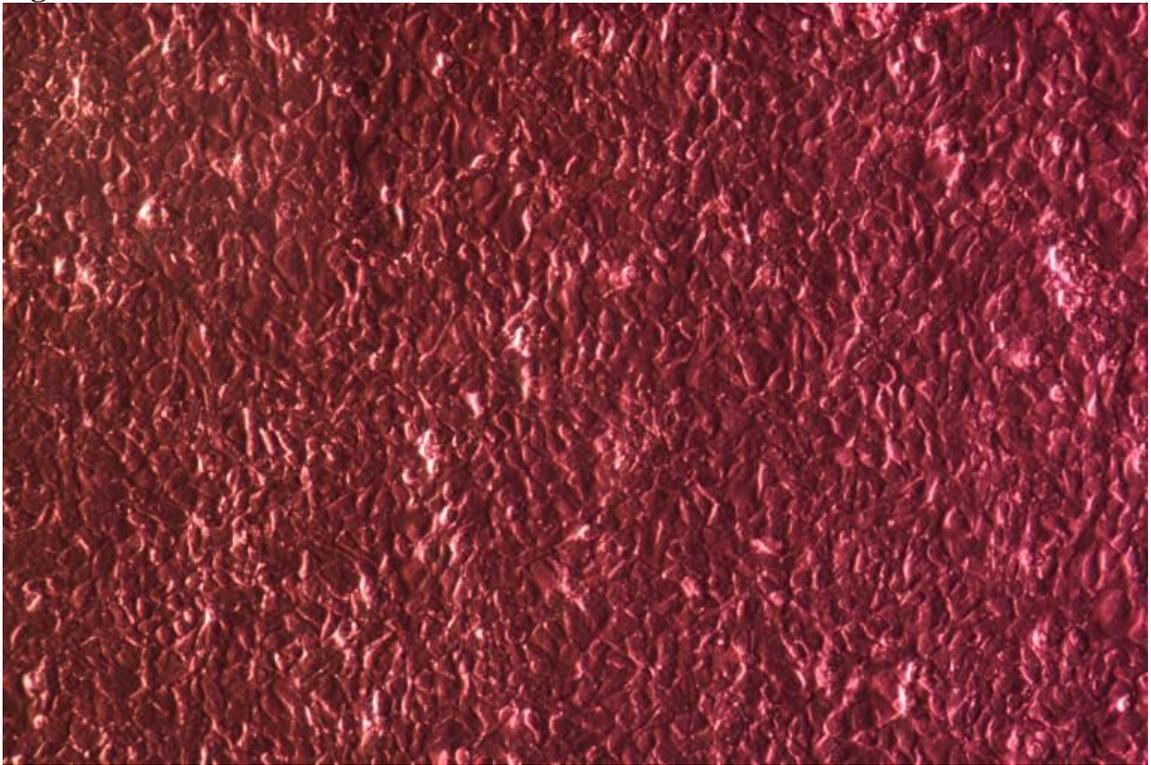


Figure 15

Figure 16

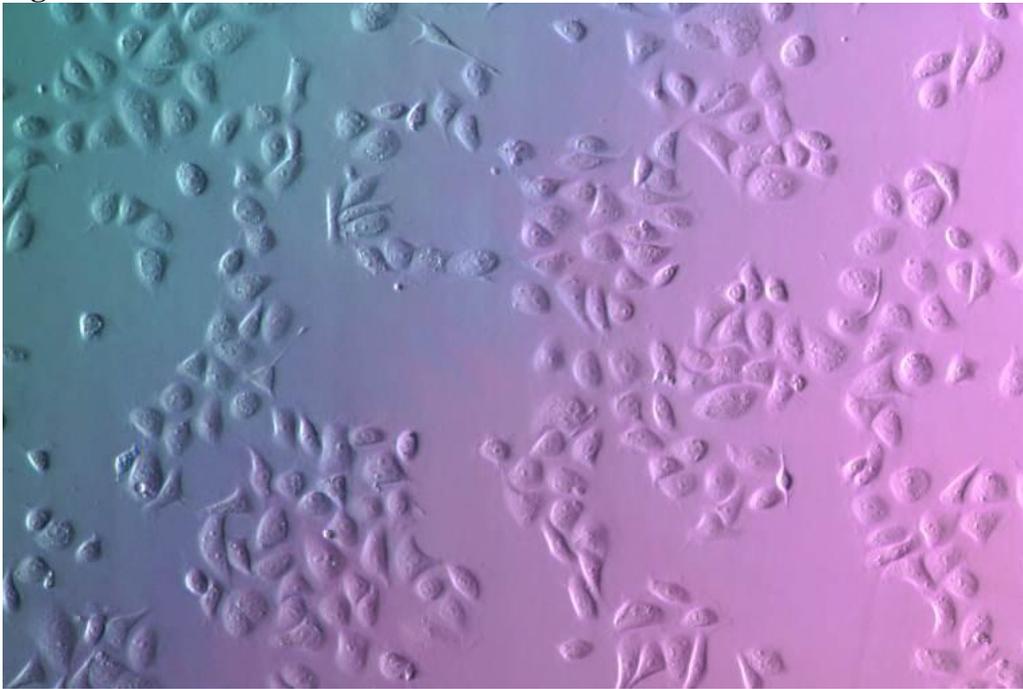


Figure 16

Figure 17

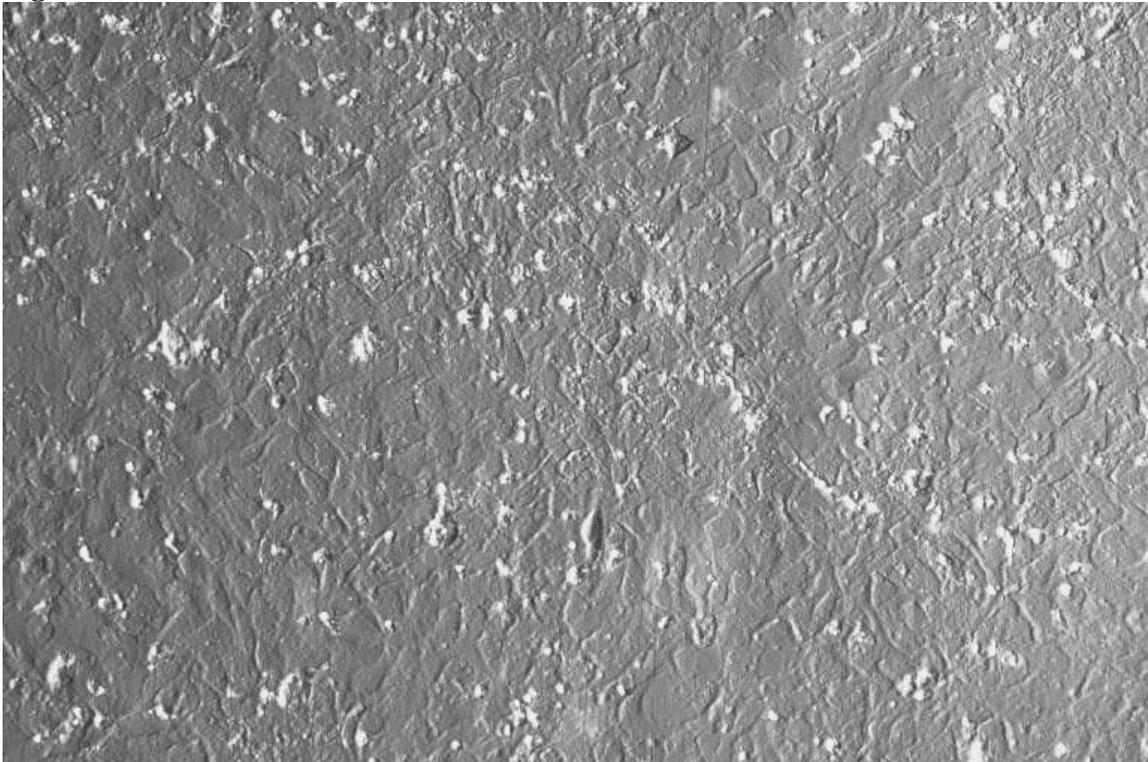


Figure 17

10.3. EMSA Studies

EMSA studies were very difficult. This is largely due to the number of steps involved in this process; even small errors could be amplified with many steps of measuring and calibration. Initial studies were complicated by poor technique. In Figure 18, one can see that while the experiment did seem to have meaning, the gel was poor and the result limited. Similarly, other gels were good, but difficulties in exposure limited the value of the final outcome. This was in part related to getting the protein loading appropriate and radiation management (see Figure 19). In other cases, simple loading of lanes could be complicated. In Figure 20, we see accumulation of tracer in lane 4, obfuscating the result.

Figure 18
ACTUAL SIZE

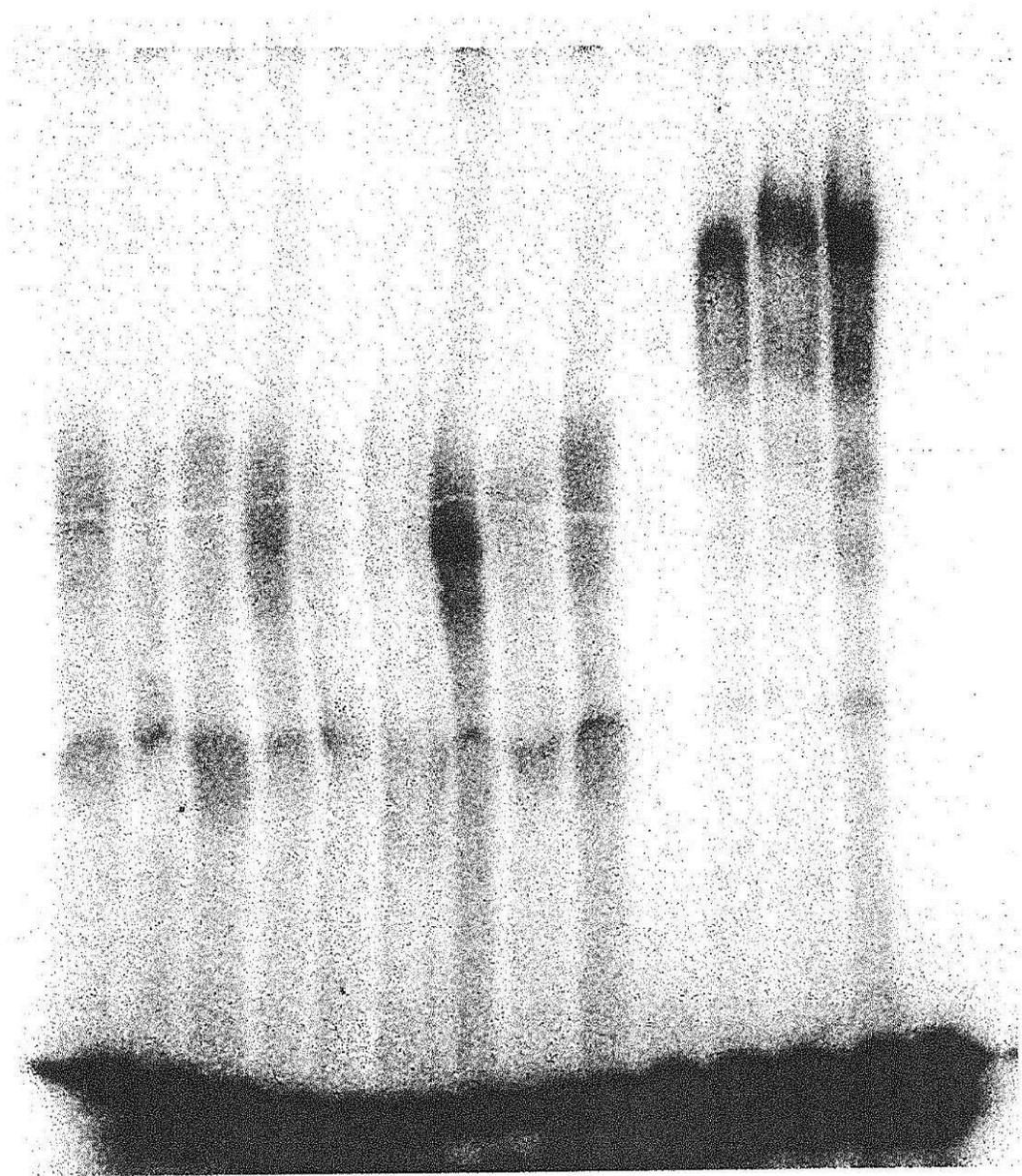


Figure 18

Figure 19

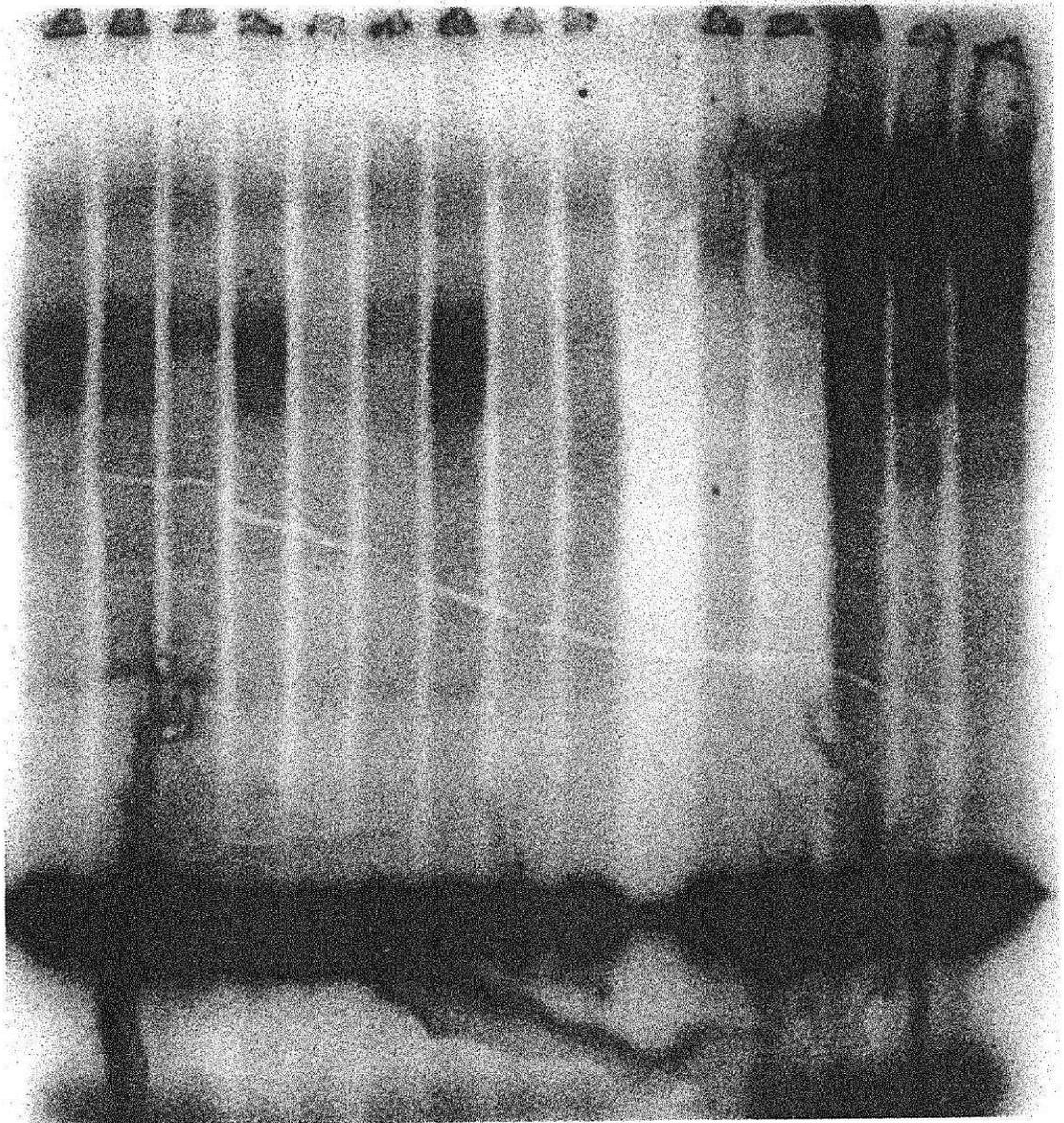


Figure 19

Figure 20

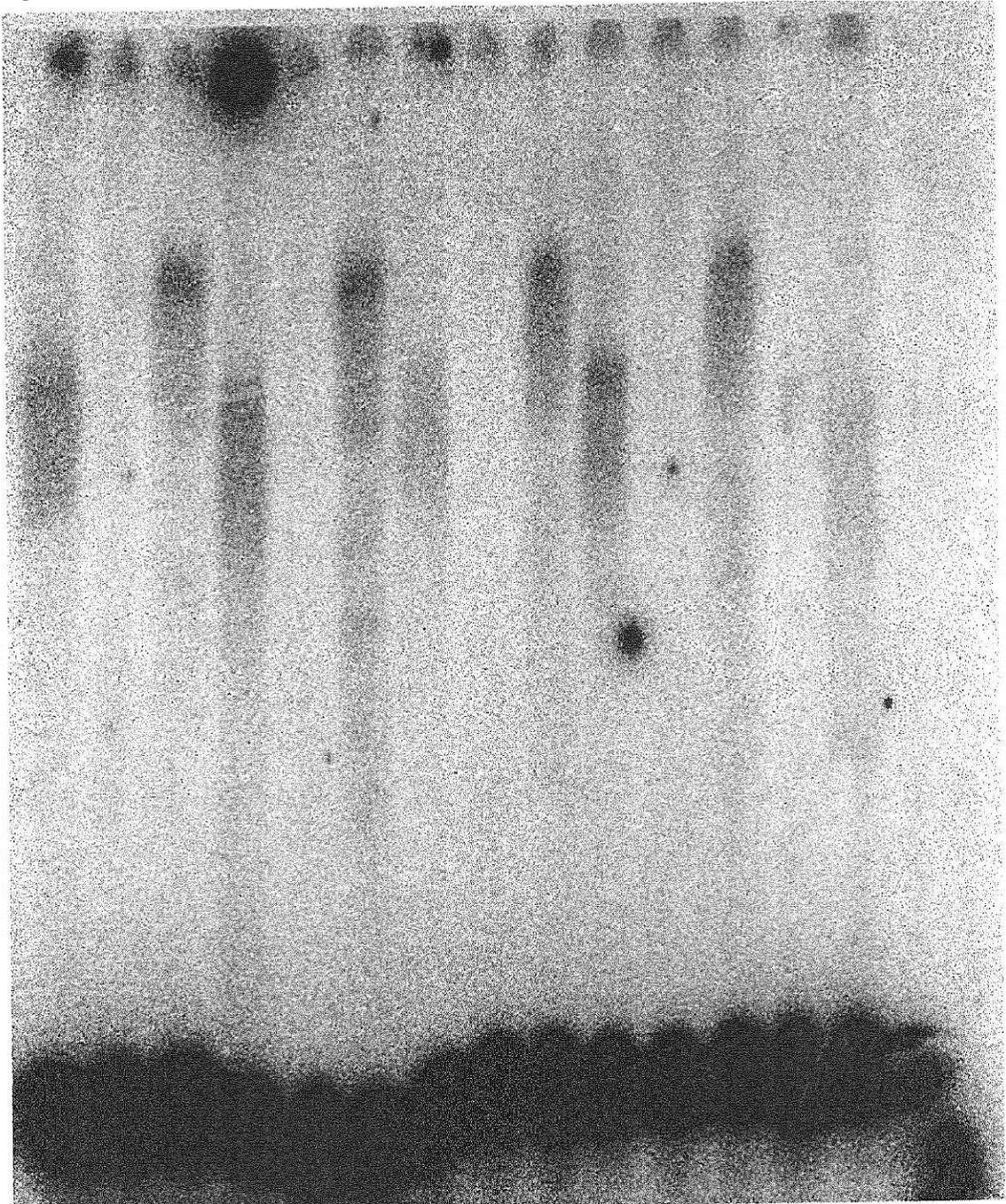


Figure 20

10.4. MTT Studies

These studies were more straightforward. The main challenge was to adjust variables including incubation time, drug concentrations, and loading cell concentrations such that controls did not grow to confluence and higher doses of treatment concentrations did not die off (see Figure 21). In Figure 22, we see the higher doses of treatments have done in the cells.

Figure 21

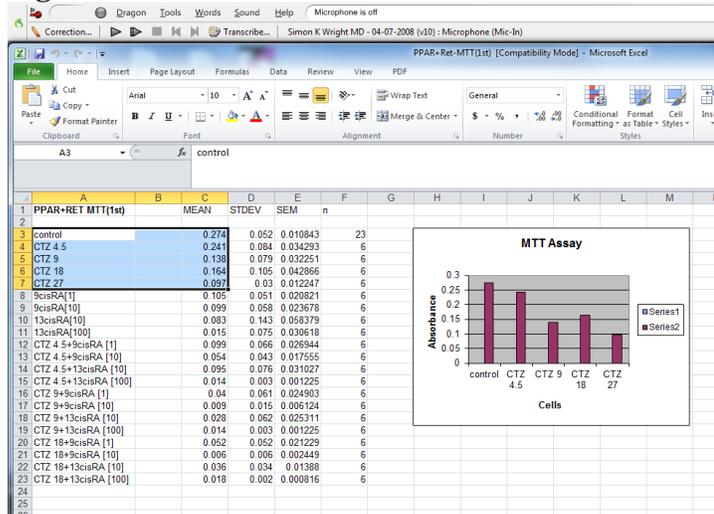


Figure 21

Figure 22

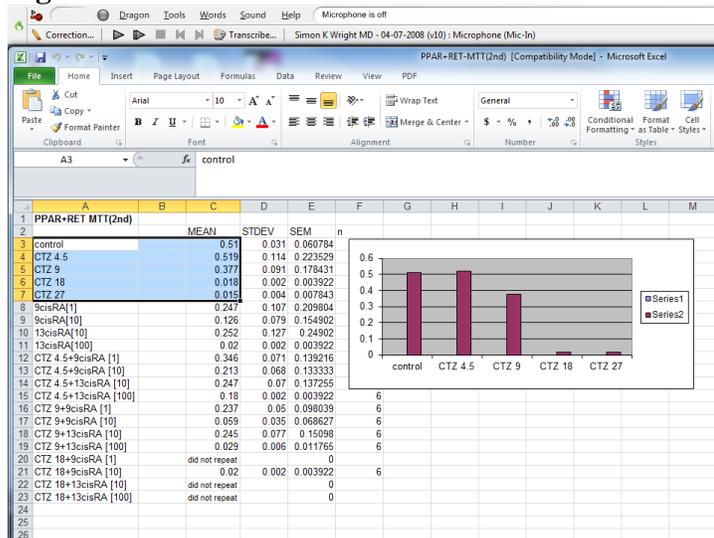


Figure 22

10.5. Reporter Gene Assays

As with many of these multi-step experiments, reporter gene assays were challenging. The luciferase assays were complicated and this was a new technique to me. Multiple failures occurred. Even good initial results were fraught with problems related to dosage and cell death (see Figure 23).

Figure 23

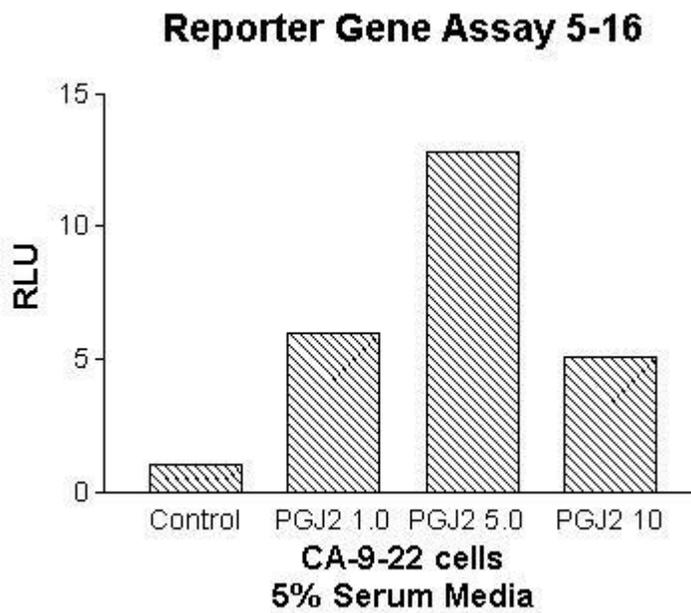


Figure 23

11.0. BIBLIOGRAPHY

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12.0. APPENDICES

12.1. Appendix A: Malignant Melanoma Studies

12.1.1. Antiproliferative and Differentiation Effects of PPAR γ Agonists on Malignant Melanoma

12.1.2. Introduction - PPAR γ and Malignant Melanoma. The presence of PPAR γ in malignant melanoma was first demonstrated by Placha, et al [332] using malignant melanoma cell lines. Investigators demonstrated the expression PPAR γ receptor RNA and protein using reverse transcription polymerase chain reaction and western blot analysis, respectively [333]. Using ciglitazone and PGJ2, authors demonstrated apoptosis and decreased proliferation.

Because previously unrecognized PPAR γ ligands have been shown to induce morphologic changes associated with differentiation in earlier experiments in our lab, as well as anti-cancer effects in other cancer models, we examined malignant melanoma cell lines and for response to activation of PPAR γ . We investigated the effect of activation of this transcription factor on growth and differentiation. Our results indicate that PPAR γ activation of cell lines with a variety of PPAR γ ligands of different chemical classes causes morphologic changes consistent with a lipogenic phenotype and differentiation, growth arrest, as well as expression of lipid-specific proteins. We conclude that PPAR γ ligands may represent a class of drugs which have value in the treatment of malignant melanoma.

12.1.3. Materials and Methods

12.1.3.1. Cell Culture: A375 and A2058 cell lines both derived from human malignant melanoma were purchased from AATC and grown at 37 C, 5% CO₂ as adherent monolayer cultures in ATCC complete growth medium: 2% Tumor Medium (Tu2%) containing a 4:1 mixture of MCDB 153 medium with 1.5 g/L sodium bicarbonate and Leibovitz's L-15 medium with 2 mM L-glutamine supplemented with 0.005 mg/ml bovine insulin, 1.68 mM CaCl₂, and 2% fetal bovine serum. Log-phase cells were routinely subcultured weekly after trypsinization.

12.1.3.2. Oil Red O Studies. Cell lines were grown to 50-60% confluency in 12-well flat bottom plates; respective ligands were added in 2% media and incubated for 3-5 days. The media was then aspirated and cells fixed in alcoholic gel according to the method of Maksem [334, 335] and plated on slides and allowed to dry overnight. ORO staining was then performed using commercial processors. Digital microphotographs were obtained using SPOT imaging software and were grossly assessed for lipid droplet uptake, morphologic changes, and ORO staining.

12.1.3.3. Cell Count Studies. A375 and A2058 cells were trypsinized during log phase growth and 400 cells per well in 5% media and allowed to attach overnight. The following morning the agents or the appropriate vehicle controls were added to the wells. After 10 days wells were rinsed with normal saline and trypsinized and counted using a

hemocytometer. The treatments were conducted in triplicate, experiments were repeated with similar results twice, and results are expressed as fractional number of cells over control.

12.1.3.4. MTT Assays. The cell proliferation rate of A375 and A2058 cells in vitro were determined by using the MTT reaction assay (Boehringer Mannheim, Indianapolis, IN). Replicate cultures of each of the cell lines were plated in 96-well, flat-bottom plates at an initial concentration of 5000 cells/well in complete media overnight. The following morning, media was replaced with serum free media containing various **PPAR γ** ligands and control containing with ETOH or DMSO alone. Cells were incubated three days prior to the lysis and assay according to manufacturer instructions using a Spectra plate spectrophotometer at 560 nm using Tecan software.

12.1.3.5. Adipophilin Immunohistochemistry. Cell lines were grown to 50-60% confluency in 12-well flat bottom plates; respective ligands were added in 2% media and incubated for 3-5 days. Cells were fixed in 10% methanol and until IHC performed. A FITC conjugated monoclonal mouse anti human adipophilin antibody was utilized (Research Diagnostics cat#RDI-PRO614102) [336] for overnight incubation. Cells were visualized using fluorescence microscopy and digital photographs taken.

12.1.4. Results

12.1.4.1. Ligands of PPAR γ Induce Lipogenic Phenotype. The effect of PPAR γ ligands on the differentiation of MM cell lines was assessed using oil-red-o staining. As shown in Figure A1, cell lines treated with PPAR γ ligands demonstrated a morphologic change characterized by the acquisition of cytoplasmic lipid droplets. At higher doses, cell rounding and decreased cell density is observed, consistent with cell death and inhibition of proliferation, respectively. Even in these cells, lipid droplets are observed. Decreased cell density was a consistent finding across cell lines and all PPAR γ ligands. Vehicle-only control cells grew to confluence.

Figure 24

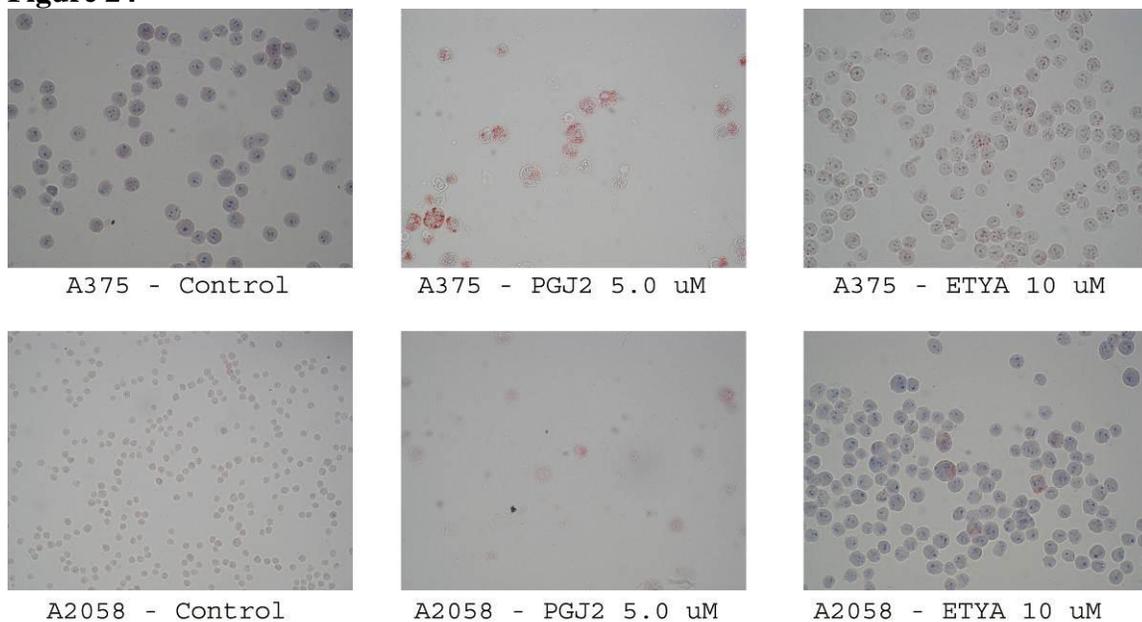


Figure A1. Oil Red O Staining. Cells were treated with respective activators, harvested and prepared in cytospin method for staining. Robust accumulation of lipid vacuoles can be seen in cytoplasm, compared to controls.

12.1.4.2. Ligands of PPAR γ Induce Growth Arrest in MM Cell Lines. The effect of PPAR γ ligands on growth of A375 and A2058 cell lines was examined using Cell Count and MTT assay. As shown in Figure A2, both cell lines demonstrated a dramatic dose-dependent decrease in cell count compared to vehicle when treated with each structurally unrelated ligand. The proposed endogenous PPAR γ ligand 15d-PGJ2 causing growth inhibition and cell death at 5 micromolar concentrations, which is similar to its EC₅₀. Each agent caused significant inhibition of proliferation followed by cell death at higher concentrations, though sensitivity to these agents was not identical for each cell line. For instance, ETYA appeared to have more efficiently at lower concentration on the A375 cells compared to the A2058 cell line. See Figure A3.

Figure 25

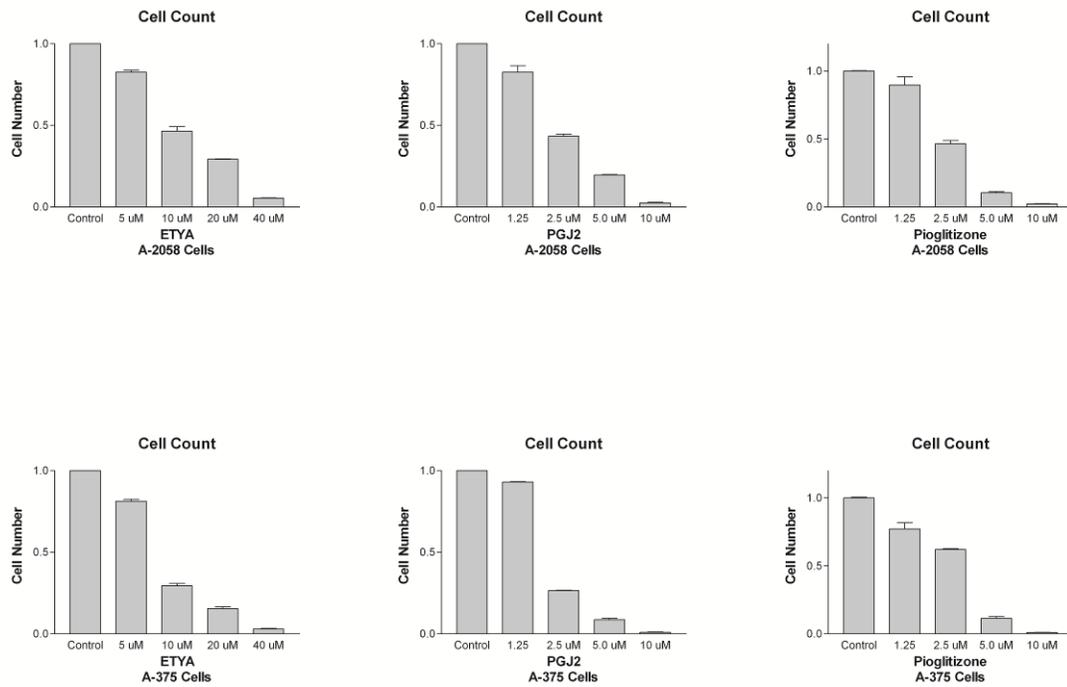


Figure A2 Cell Count Assays. Log-growth cells were harvested and treated in 2% serum-containing media for 40 hours, then harvested and counted. Six samples per group and experiment repeated in triplicate. Experimental groups represented as percent of control.

Figure 26

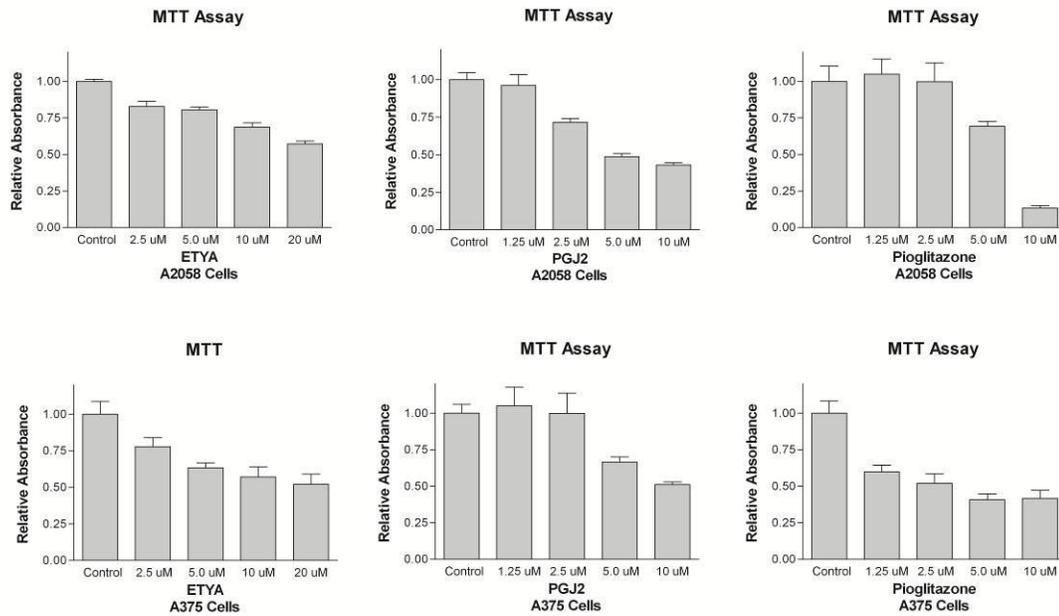


Figure A3. MTT Assays. Log-growth cells were harvested and treated in 2% serum-containing media for 40 hours, then prepared for MTT assay. Six samples per group and experiment repeated in triplicate. Experimental groups represented as percent of control.

12.1.4.3. Ligands of PPAR γ Induce Adipophilin Production in MM Cell Lines. The effect of PPAR γ ligands on lipid differentiation of A375 and A2058 cell lines was examined using IHC for adipophilin. Adipophilin is a lipid biomarker associated with lipid droplet formation. In cells treated with PPAR γ agonists, lipid droplet formation was observed by ORO staining. To confirm lipid droplet formation, immunohistochemistry for adipophilin was performed. This demonstrated distribution of fluorescence around lipid droplets in the perimeter of the cells with little or no staining elsewhere in the cells, Figure A4. This occurred with ligand agonism at concentrations in keeping with the activation of cells and occurred with each PPAR γ activator studies.

Figure 27

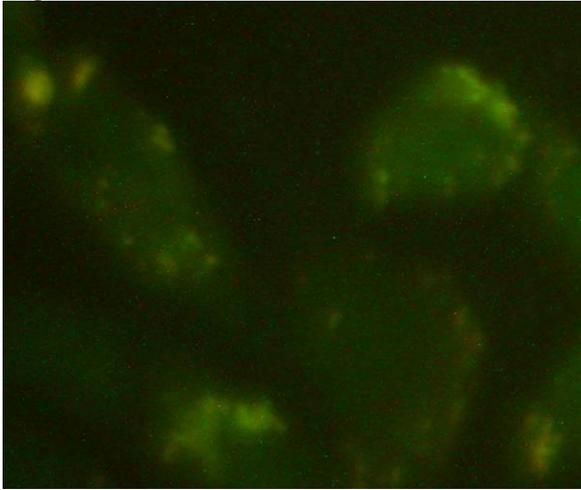


Figure A4. Adipophilin IHC. Log-growth cells were plated in slide flasks and allowed to grow to 30% confluence, then treated with PPAR γ agonists.

12.1.5. Discussion

Malignant melanoma is an aggressive form of skin cancer causing the majority of death from skin cancer. The incidence of this disorder is on the rise in Western populations. During the past few decades, the incidence of cutaneous malignant melanoma has been rising in both sexes in almost all developed countries [337].

While the prognosis for early stage cutaneous malignant melanoma is good, a 5 year survival for patients with lymph node metastasis is only 54%. For disseminated malignant melanoma, the prognosis is dismal, with a 5 year survival of 6% and median survival duration of 7.5 months [338, 339]. While melanoma progression is well-defined in its clinical, histopathological, and biological aspects, the understanding of molecular mechanisms involved and genetic markers associated with metastatic dissemination are early in its development. Novel therapeutic modalities are greatly needed for the management of this disorder. Alternative approaches will likely be necessary to achieve additional therapeutic gain in the treatment of this aggressive neoplasm.

Targeted therapy through PPAR γ activation is supported by prior studies demonstrating anti-proliferative and pro-apoptotic effects of PPAR γ activation in malignant melanoma. Prache, et al. demonstrated PPAR γ expression and inhibition of cell proliferation and induction of apoptosis in MM cell lines. This was confirmed by Freudlsperger, et al. [333] who demonstrated an anti-proliferative effect of four TZD drugs.

In the present study, we also observe decreased. This study demonstrates the novel observation of lipid accumulation as demonstrated through oil-red-O staining and through biomarker assessment. The phenotypic shift from melanocyte to lipid-accumulation including adipophilin production and lipid vacuole accumulation represents complex intracellular trafficking in keeping with a shift in differentiation. Similar studies (in press) in squamous cell carcinoma have demonstrated similar results with the additional

finding of AP2 production, a marker of terminal differentiation. This implicates the activity of at least two major anti-cancer effects of TZD drugs, namely antiproliferation and differentiation. The role of apoptosis was not studied, though Freudlsperger did observe apoptosis in MM cell lines in response to treatment with rosiglitazone.

Additional studies that would further define the events underlying the anti-proliferative and differentiation observations include apoptosis assay, AP2 assay, as well as a panel of ELISA studies investigating markers of invasion, such as proteases.

In summary, the TZD drugs are already in clinical use as anti-diabetic drugs, which make them attractive from a translational standpoint. This study demonstrates both an anti-proliferative effect of PPAR γ activation as well as evidence of lipid differentiation through lipid accumulation and functional lipid-protein associated in through the biomarker adipophilin. Further investigation of PPAR γ activation as a potential therapeutic target in the management of MM should be undertaken.

Appendix B: Peroxisome Proliferator-Activated Receptors (PPAR) in Adenocarcinoma Cells

Simon K. Wright, Kenning Anderson, Lindsay Miller, Heather Zahn, Jules Harris, Frank Ondrey.

12.1.6. Introduction. Prostate cancer is the second leading cause of death among aging men in the United States and is the most common form of cancer [340]. Pharmacologic activation and inhibition of steroid metabolism has been a tissue-specific treatment for a variety of blood borne and solid tumor malignancies. Androgen-dependent growth of diseases like prostate cancer can be specifically antagonized with acceptable outcomes. However, prognosis usually worsens when tumors gain steroid-independent growth [341]. It is possible that multiple nuclear orphan receptor classes participate in interactions allowing tumor growth, and that overdriving specific differentiation-associated pathways in the nuclear orphan hormone receptor family may result in therapeutic gain. Studies have indicated that PPAR gamma may be a potential target for prostate cancer treatment [342, 343]. In the present study it was hypothesized that these pathways might still be therapeutically exploited in tumors exhibiting steroid-independent growth. Androgen-independent PC-3 cells were grown and treated with several PPAR agonists (ETYA, ciglitazone, PGJ2) and retinoids (9 and 13 cis-retinoic acid) and examined for changes in cell proliferation and functional activation of PPAR reporter genes. Additionally, nuclear extracts were examined for complexes with the capability of binding double-stranded oligonucleotides containing PPAR recognition sequences exhibiting a steroid DR-1 sequence (EMSA assay) before and after treatment. Other experiments examined ultrastructural changes on EM after treatment with one of the PPAR agonists (ETYA). All of the agonists tested significantly increased Bgal-corrected luciferase activity by 1.5 to twenty fold in several experiments. EMSA complexes in cells treated with PPAR agonists contained heterodimers consisting of at least PPAR γ and RXR elements on supershift analysis. Complexes containing PPARs were observable at baseline conditions as well. On MTT assay, significant dose-dependent decreases in cell proliferation were also observed. Several ultrastructural changes were noted after ETYA treatment, which included 60% decrease in mitochondrial numbers as well as myelin figures and osmiophilic lipofuscin which both indicate increases in cellular lipid. We conclude that pre-existing PPARs can be activated in prostate PC-3 cells with specific agonists of both PPARs and RXRs, and their activation precedes morphologic change and decreased proliferation. We conclude that PPAR pathways can be activated in steroid-independent tumor growth and may represent a novel target for intervention.

12.1.7. Materials and Methods.

12.1.7.1. Cell Culture. Androgen independent PC-3 prostate cancer cells were grown in RPMI 1640 Media supplemented with 2mM glutamine and 10% defined FBS incubated at 37⁰ C 5% CO₂. The cell lines were maintained in continuous culture and subcultured weekly.

12.1.7.2. MTT assays: Log-phase PC-3 cells were gently centrifuged at 125xg, and placed into 96 well microtiter plates in 10% FCS containing media. ETYA, ciglitazone,

and PGJ2 or its carrier (DMSO) was added at various concentrations by serial dilution. After incubation for 72 hours, MTT reagent (Boehringer Mannheim, Indianapolis, IN) was added to all wells for 4 hours. Overnight incubation in the kit-supplied lysis buffer was performed. The assays were read the following day at 560 nM on an Elisa Plate reader.

12.1.7.3. EMSA. PC-3 cells were grown to near confluence under standard conditions and with respective concentrations of ETYA, ciglitazone (CTZ), or PGJ-2 for 40 hours. Cells were then trypsinized, centrifuged and washed with normal saline + 0.5 mM PMSF + 1 µg/ml Leupeptin. Cytosolic and nuclear fractions were separated and protein concentration was determined by Bradford assay. Five µg of nuclear protein was incubated with P-32 labelled oligonucleotide probe (hormone response element of CYP-4A1 gene) at 50,000 cpm for ten minutes at room temperature in binding buffer (10mM Tris HCl pH 8.0, 150 mM KCl, 6% Glycerol, 0.05% NP-40, 1 mM DTT, 100 ng/ µ L poly-DIDC). Cold and mutant competition lanes were pre-incubated with respective un-labelled probes at 100x for 30 minutes on ice. Supershift lanes were pre-incubated with 4 µg mouse anti-human PPAR γ antibody (Santa Cruz) overnight. Samples were then loaded into a 5% polyacrylamide gel in 0.5X TBE buffer and run at 200V for 90 minutes. Gels were dried then exposed to scintillation sheets overnight, then read on an imager (Cyclone Optiquant, Packard Systems). Computer analysis performed on Optiquant image analysis.

12.1.7.4. Transient Transfections/luciferase Assays. PC-3 cell lines were transiently transfected with a thymidine kinase luciferase containing reporter plasmid with a PPRE, kindly gifted from Ron Evans; TK-PPREx3-Luc (PPRE x 3 (5'-GTTCGACAGGGGACCAGGACAAAGG-TCACGTTCGGGAGTCGAC), three copies)]. Cells at 70% confluency were exposed to 2 µg/ml TK-PPREx3-Luc reporter construct plasmid and co-transfected with 0.4 µg/ml beta-Gal using Lipofectamine for 4 hours. Cells were incubated in complete media for 24 hours, then harvested. Relative luciferase activity was assayed with a Dual-Light reporter gene system (Tropix, Bedford, MA) as per kit instructions using a luminometer.

12.1.7.5. Electron Microscopy. PC-3 cells were grown under standard conditions and treated with ETYA and vehicle alone. Cells were then fixed and dehydrated and transmission EM performed using standard techniques.

12.1.8. Results. Proliferation of both cell lines was decreased > 50% in a dose-dependent fashion both with PPAR agonist and RXR agonist treatment. (see Figure B2) Reporter gene assays using the TK-PPREx3-Luc reporter element, which represents the hormone response element for PPARs, demonstrate functional activation of the gene in a dose dependent manner. (see Figure B3) Nuclear proteins composed of PPAR γ and Retinoid X Receptor (RXR) heterodimers specifically bound PPAR γ transcription recognition sites and were induced by ciglitazone (see Figure B4), as well as ETYA and PGJ2 (not shown). Supershift assays demonstrate at least two subunits, including RXR-alpha and PPAR γ proteins.

Treatment of cells with PPAR agonists induced ultrastructural changes, including 60% decrease in mitochondria numbers in addition to formation of myelin figures and osmiophilic lipofuscin, two markers of cellular lipid. (see Figure B5)

Figure 28

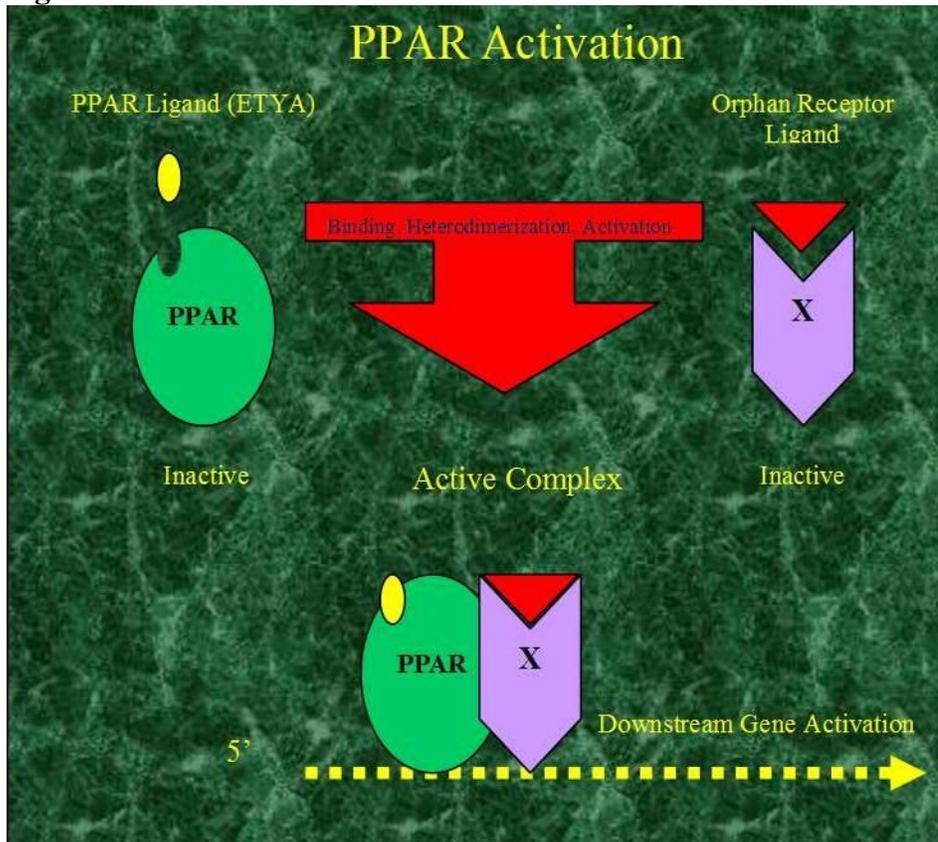
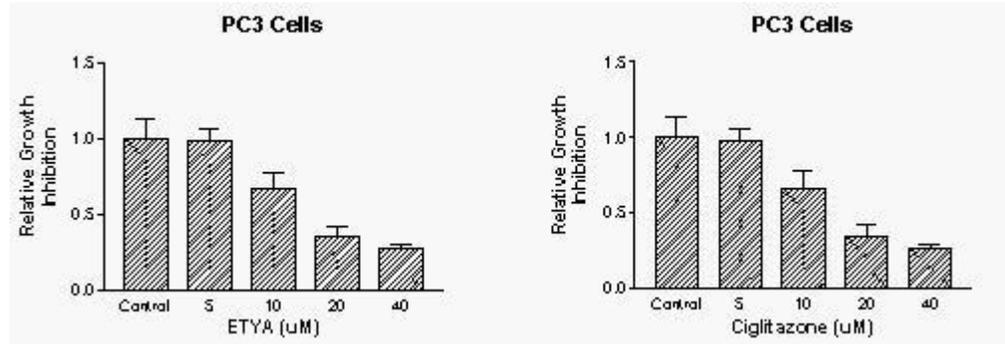


Figure B1. PPAR nuclear hormone receptor complex composed of heterodimer consisting of PPAR and RXR subunits.

Figure 29

a.



b.

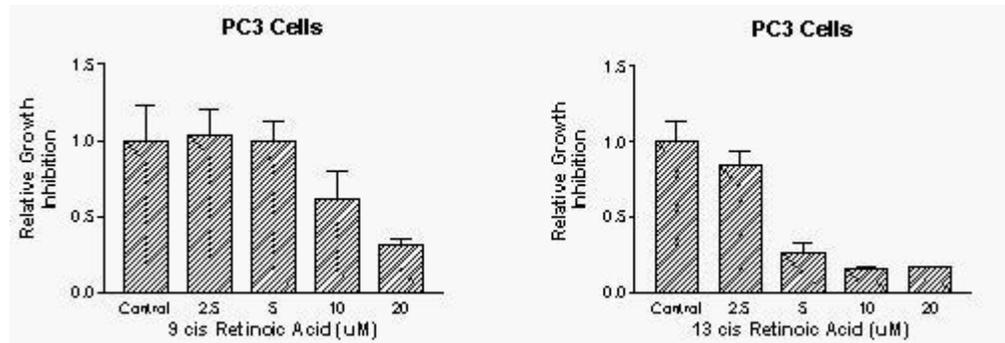


Figure B2. MTT Assays. Log phase growth steroid-independent PC-3 cells were grown to subconfluence and treated for 3-5 days with various PPAR γ or RXR agonists.

Figure 30

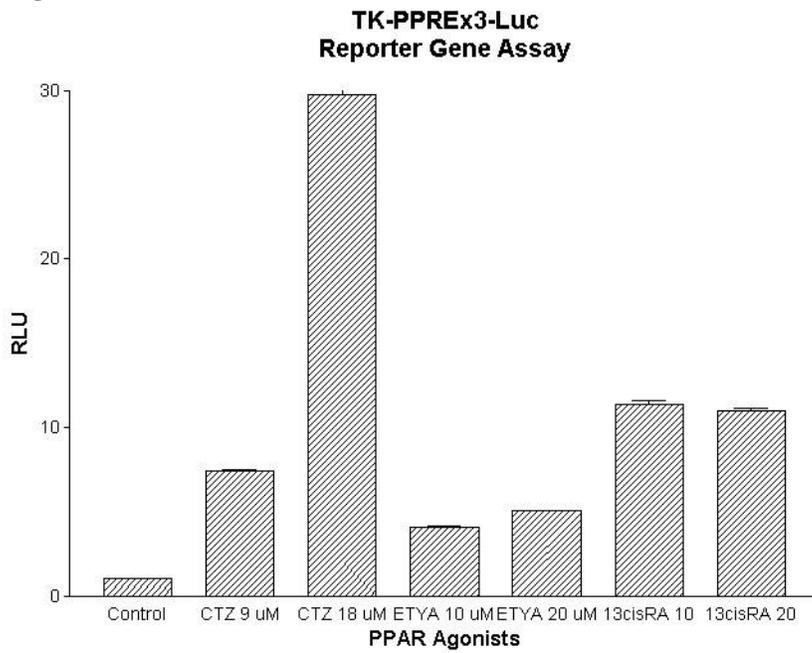


Figure B3. Reporter gene assay. PC-3 cells were co-transfected with B-gal and the TK-PPREx3-Luc reporter plasmids and incubated in serum free conditions with the respective PPAR or RXR activator.

Figure 31

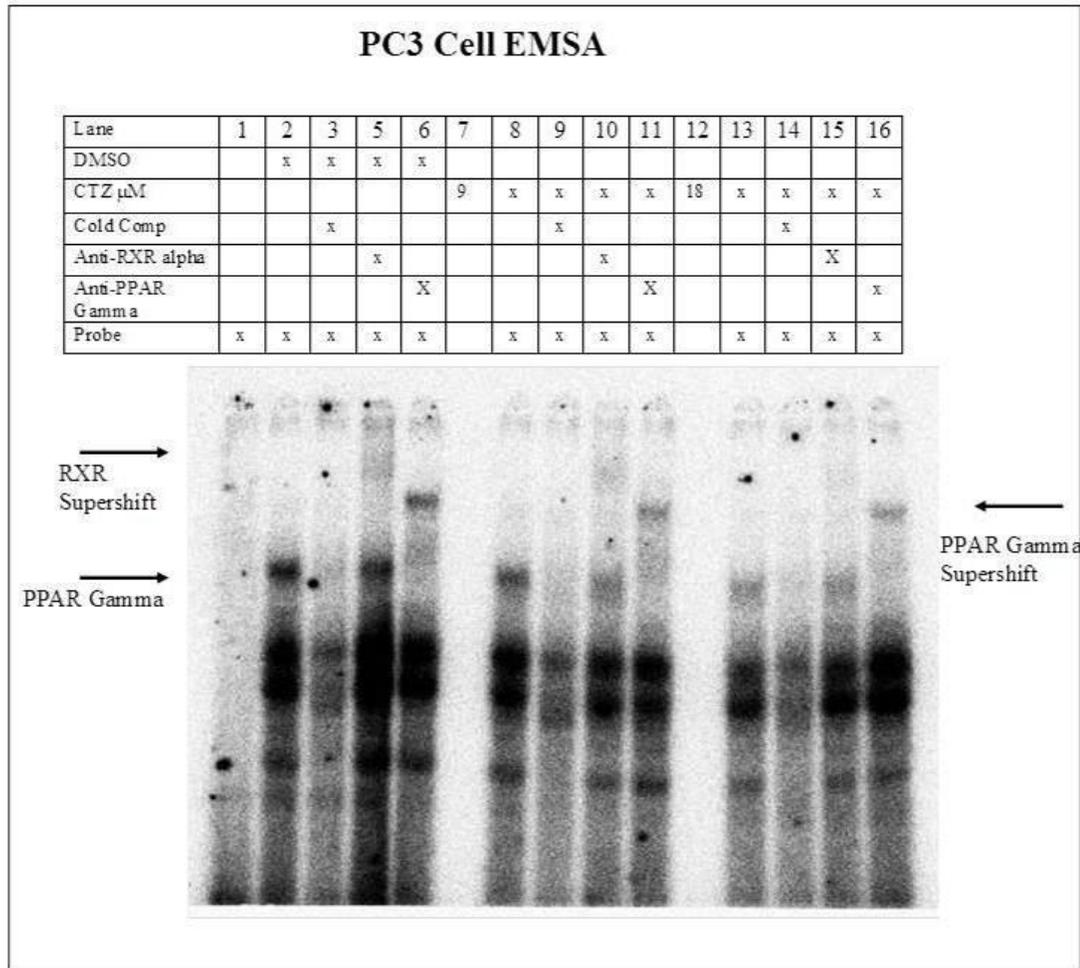


Figure B4. EMSA. Lanes 1, free probe; Lanes 2-6, vehicle; lanes 8-11, treated with ciglitazone 9 μ M; lanes 13-16 ciglitazone 18. Nuclear extracts from control and treated cells were incubated with oligonucleotide probes corresponding to the CYP4A1 hormone response element, which is specific for PPAR γ . Cold competition lanes were run for each group.

Figure 32

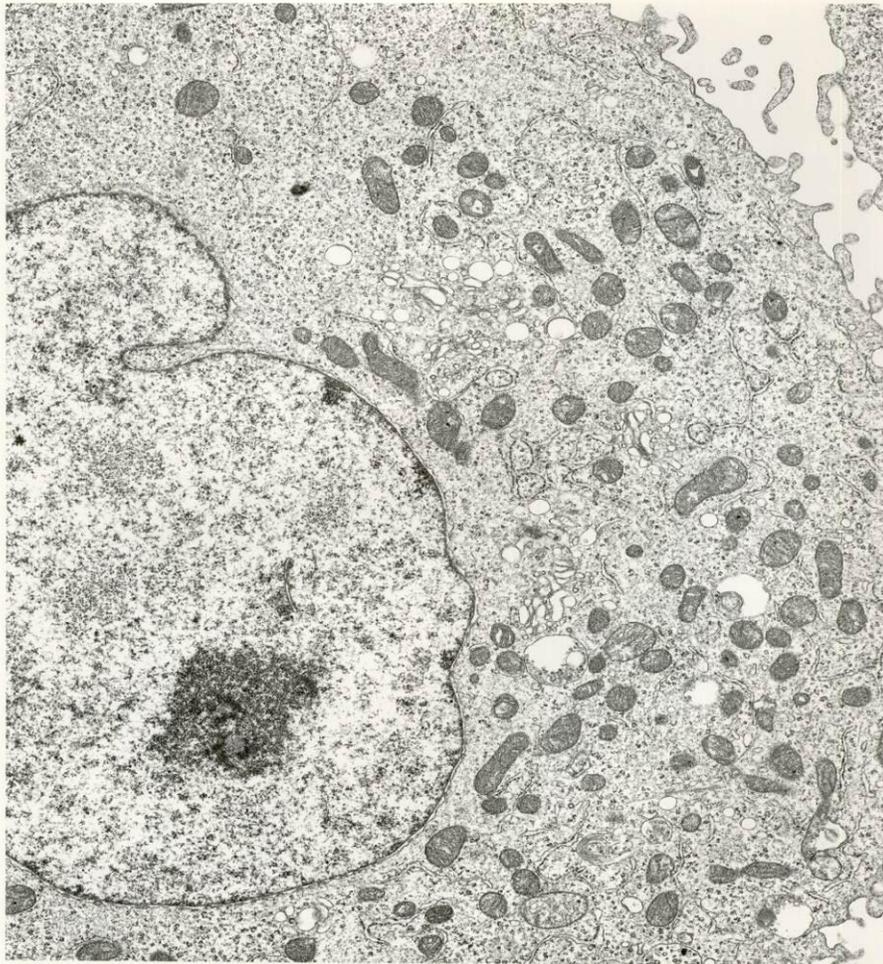


Figure B5a. Control PC-3 cell, 14,000 X. Visible are mitochondrial cristea, no lipid droplets.

Figure 33

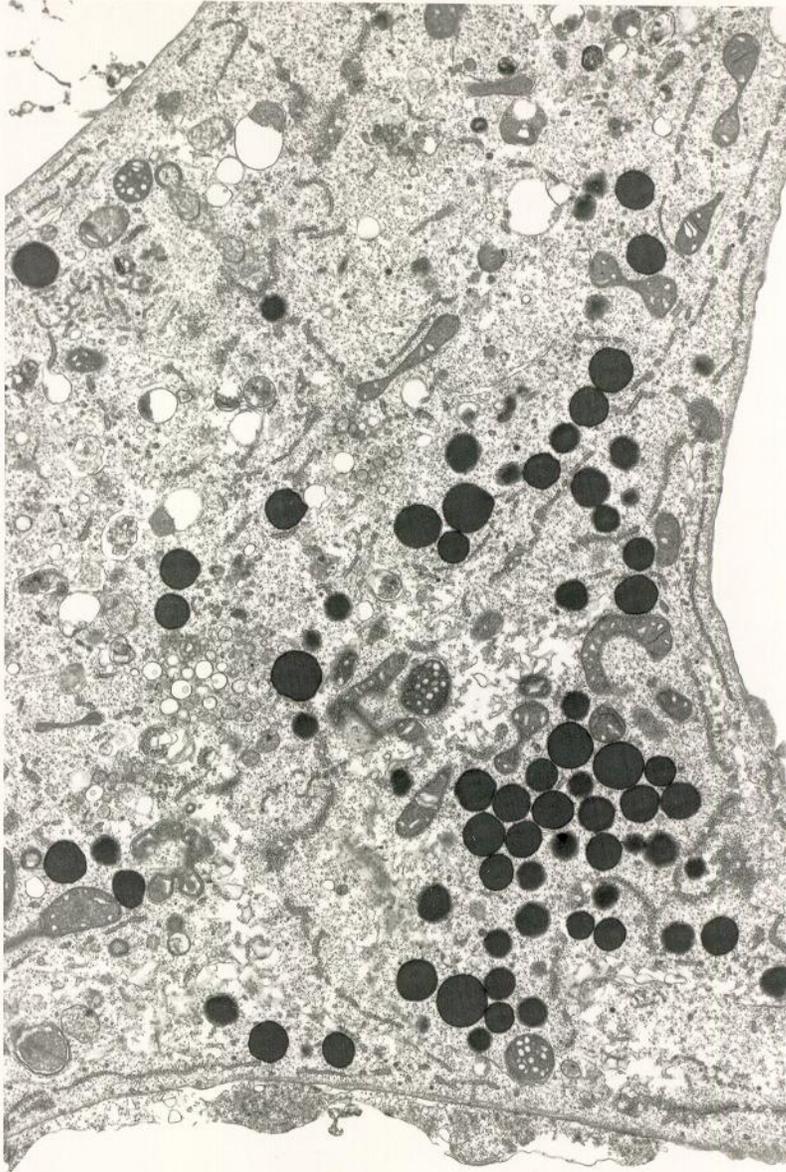


Figure B5b PC-3 cells treated 48 h with ETYA 40 uM in serum containing conditions. Note damage to mitochondria, increased droplets without encircling membrane. 9240 X.