Retinoids Augment Thiazolidinedione PPARγ Activation in Oral Cancer Cells

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Abstract. *Background/Aim: Head and neck squamous cell carcinoma affects nearly 500,000 people annually. Augmenting PPARγ functional activation is linked with multiple anti-carcinogenic processes in aerodigestive cell lines and animal models. PPARγ/RXRα heterodimers may be key partners in this activation. Materials and Methods: CA 9-22 and NA cell lines were treated with the PPARγ agonist ciglitazone and/or the RXRα agonist 9-cis-retinoic acid. PPARγ functional activation, cellular proliferation, apoptosis activity, and phenotype were subsequently analyzed. Results: Ciglitazone and 9-cis-retinoic acid independently activated PPARγ and down-regulated the carcinogenic phenotype in vitro. Combination treatment significantly augmented these effects, further decreasing proliferation (p<0.0001), and increasing PPARγ functional activation (p<0.0001), apoptosis (p<0.05), and adipocyte differentiation markers (p<0.0001). Conclusion: The efficacy of the combination of ciglitazone and 9-cis-retinoic acid afforded lowering treatment concentrations while maintaining desired therapeutic outcomes, optimistically supporting the feasibility and practicality of this novel treatment option.*

Head and neck squamous cell carcinoma (HNSCC) affects nearly 500,000 individuals worldwide every year (1). The 20-50% survival rate of stage III and IV disease has remained stagnant over multiple decades despite advances in research and multi-modality treatment protocols (1, 2). This illuminates the imperative need for novel treatment options. Nuclear hormone receptor targeting has revealed its therapeutic

potential in a variety of cancers (3). A class of nuclear receptors, peroxisome proliferator-activated receptors (PPAR), were originally recognized for their role in regulating lipid and glucose metabolism (4, 5). These are therapeutic targets of interest with respect to HNSCC. An isoform of the PPAR family, PPARγ, is an intriguing target; initially described as an adipocyte differentiation transcription factor (6, 7). Cancer cells are characterized by the lack of differentiation, thus, attempting to induce differentiation may be exploitable as a treatment strategy. Differentiation therapy has previously demonstrated value as a novel treatment strategy for non-small cell lung cancer (8, 9), as well as for acute promyelocytic leukemia (10, 11). Research has shown that PPARγ can form a heterodimer with retinoic X receptor alpha (RXRα) and transcriptionally activate down-stream genes (5, 7). In lipid biology, this process has directed malignant precursor cells into non-malignant adipocytes (12). Once activated, it has been postulated that the PPARγ/RXRα heterodimer transcription factor may regulate multiple pathways with the following downstream anti-carcinogenic effects: decreased proliferation, decreased angiogenesis, and increased apoptosis (13). Research has demonstrated that cellular dysfunction involving this pathway, adversely stunting PPARγ expression, is linked to multiple cancerous etiologies including colorectal carcinomas (14) and poorly differentiated adenocarcinoma (5, 15).

Research has also demonstrated that thiazolidinedione (TZD) drugs such as pioglitazone, rosiglitazone, troglitazone, and ciglitazone, conventionally used to treat type II diabetes mellitus, can functionally activate PPARγ pathways, inducing the aforementioned anti-carcinogenic effects (8). Epidemiological analysis of 85,000 diabetic patients demonstrated that those prescribed thiazolidinedione drugs had a 33% decreased prevalence of aerodigestive lung cancer, another tumor type associated with tobacco use, when compared to a control population (16). Previous clinical trials assessing thiazolidinedione treatments for liposarcoma (17), breast (18), and prostate cancer (19) are also encompassed in this developing field. There is multidisciplinary evidence for attempting to exploit this pathway for cancer treatment and prevention.

With respect to head and neck squamous malignancies, previous studies have demonstrated that PPARγ expression was suppressed in comparison to normal squamous lining tissues which were not cancerous, accentuating the possibility that induction of PPARγ might be a treatment strategy to restore cancer cell homeostasis to a more normal phenotype (5, 7, 20). Furthermore, there was a dose dependent decrease in cell proliferation and clonogenicity of oral cancer cells in response to several classes of PPAR agonists (20).

Interventions with similar PPARγ agonists have documented synergistic effects when combined with conventional chemotherapeutic agents (21, 22). As RXRα is a principal component of the heterodimer with PPARγ (13), a retinoic acid could augment PPARγ activity when combined with another transcription factor agonist such as a thiazolidinedione. However, the historic implementations of retinoic acids for differentiation therapy have been limited due to the toxic effects of retinoids and the reversal of effects upon cessation of treatment (23, 24). The aims of this study were to examine the activity of the RXR agonist 9-*cis*-retinoic acid (9cRA), as it is FDA approved and has demonstrated to be better tolerated in clinical populations (25), and the preclinical thiazolidinedione ciglitazone (CTZ) in an attempt to examine the feasibility of activating both RXRα and PPARγ as a combination strategy for head and neck cancer treatment or prevention.

Materials and Methods

Cell culture. Mycoplasma free CA 9-22 and NA aerodigestive cell lines were cultured at 37˚C, 5% CO_2 as adherent monolayer cultures in RPMI 1640 Media supplemented with 2mM Glutamine, 10% heat-inactivated FBS (ThermoFisher, Grand Island, NY, USA) 50 U/ml Penicillin, and 50 μg/ml streptomycin (26). Log-phase cells were routinely subcultured weekly *via* trypsinization.

Reagents. Ciglitazone (CTZ) was purchased from Biomol (Plymouth Meeting, PA, USA), 9-*cis*-retinoic acid (9cRA) from Millipore-Sigma (St. Louis, MO, USA).

Luciferase reporter gene assays. PPARγ ligand-mediated transcriptional activation was analyzed *via* luciferase reporter gene assay performed as we have previously published (20). Human oral squamous cell lines CA 9-22 and NA were transiently infected with thymidine kinase luciferase-containing reporter plasmid for the PPARγ-dependent gene acyl-CoA oxidase TK-PPREx3 (kindly gifted by Ron Evans). This contains TK-PPREx3-LUC [PPRE x 3(5';-GTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCG AC), three copies], and when PPARγ binds to these PPAR response elements, the reporter construct produces luciferase. Cells at 60-80% confluence were co-transfected with 2 μg/ml TK-PPREx3-Luc reporter plasmid and 0.4 μg/ml β-Galactosidase containing DNA reporter *via* cationic lipid transfection (Lipofectamine at 10 μg/ml in Opti-MEM (ThermoFisher) for 4 h. Cell lines were then treated for 24 h with CTZ and/or 9cRA. Relative luciferase activity (RLU) was assayed with the Dual-Light reporter gene assay system (ThermoFisher) using the Tropix model TR717 dual injection plate luminometer (Berthold Technologies, Oak Ridge, TN, USA) and luciferase was normalized to βGal. Three assays were performed in triplicate wells for a total of nine replicates per data point.

MTT cell proliferation assay. Cell proliferation was determined using the MTT reaction assay (Boehringer Mannheim, Indianapolis, IN, USA). CA 9-22 and NA cells at a density of 7.5×10^4 cells/well were seeded in 96-well plates (Corning, Corning, NY, USA). Cell lines were treated with CTZ and/or 9cRA in serum free media on day 0. On day 0, 1, 3 and 5, 0.5 mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide were added to the culture media and incubated at 37˚C for 4 h. Mitochondrial dehydrogenases of live cells convert MTT to a water insoluble purple formazan, which we then solubilized in isopropyl alcohol/DMSO. The absorbance was analyzed at 560 nm on a Tecan model 530 plate spectrophotometer using Tecan software (Tecan, Morrisville, NC, USA). Six replicates of each test group were assayed.

Probe preparation. The RXR (DR-1) probe and the mutant oligonucleotide containing two "GT"→"CA" substitutions in the RXR binding motif, was obtained commercially and contained the following sequence: 5';-AGC TTC AGG TCA GAG GTC AGA GAG CT-3'; (sc-2547, Santa Cruz Biotech, Inc., Dallas, TX, USA), 5';-AGC TTC AGC ACA GAG CAC AGA GAG CT-3'; (sc-2548, Santa Cruz Biotech, Inc.), respectively. The Cyp4A1 probe was manufactured by GibcoBRL, part of ThermoFisher, as a sense and anti-sense oligonucleotide. The Cyp4A1 probe contained the published Cyp4A1sequence, 5';-TGA AAC TAG GGT AAA GTT CA-3'; Cyp4A1 DNA strands were annealed by heating in annealing buffer (0.1 mM Tris-HCl, 1.0 M NaCl, 1x React2 (GibcoBRL/ThermoFisher), 300 pM oligonucleotide) to 60˚C, and then cooled to room temperature. End labeling with T4 polynucleotide kinase (Promega, Madison, WI, USA) and γ-32-ATP (6000 Ci/mmol, Amersham, Arlington Heights, IL, USA). Double stranded DNA oligonucleotide probes for OCT-1 were obtained commercially (Promega).

EMSA binding reactions and shift assays. Electromobility shift assay (EMSA) binding reactions consisted of 5 μg of nuclear extract protein and performed identical to previous studies (8). Gels were imaged with phosphor imaging with a Packard Cyclone Phosphorimager utilizing multipurpose imaging screens and analyzed by Optiquant software (Packard Technologies, Downers Grove, IL, USA). Each EMSA reaction was performed with at least two separate nuclear extract preparations. Supershift reactions utilized 1 μg of antibody for RXRα (sc-553, Santa Cruz) or PPARγ (sc-7273, Santa Cruz) added 12 h prior to binding reactions with the labeled probes. All reactions carried out at 4˚C and densitometry analysis was performed with Optiquant software.

Caspase 3/7 assay. Apoptosis was analyzed by monitoring caspase 3/7 activity. NA and CA 9-22 cells were plated at 10×10^3 cells/well density in opaque clear bottom 96 well plates. Treatments, alone and in combination, were added the following day. After 24 hours of treatment the caspase 3/7 activity was assayed *via* Caspase-Glo® 3/7 assay (Promega), a luminescent assay which provides a signal proportional to caspase 3/7 activity. The single Caspase-Glo® 3/7 reagent lyses the cells and caspase cleavage of the substrate results in free aminoluciferin, which is consumed by the luciferase, creating a glow-luminescent signal proportional to caspase-3/7 activity.
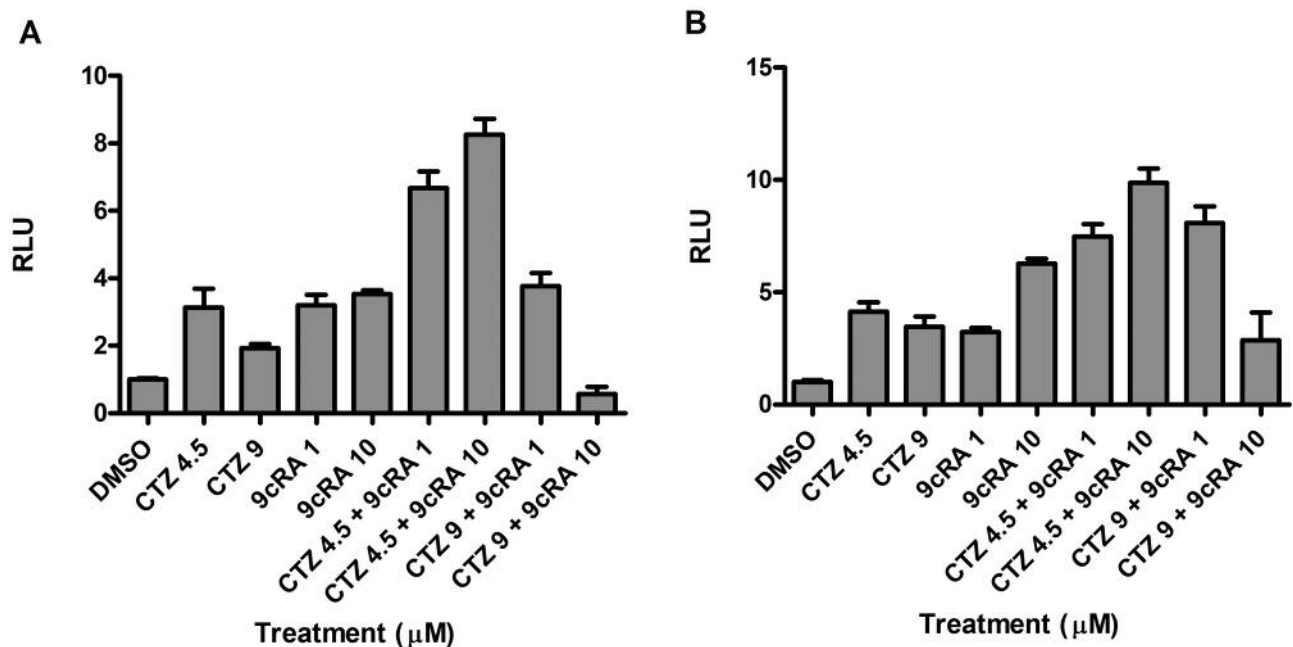
Figure 1. *Reporter gene activity in NA and CA 9-22 cell lines treated with ciglitazone (CTZ) and/or 9-cis-retinoic acid (9cRA) for 24 h. RLU (y-axis) is representative of relative luciferase activity (RLU), normalized to βGal. Each column is the average of nine data points. Graph A represents the NA cell line. Graph B represents the CA 9-22 cell line. One-way ANOVAs employed a Tukey post-test, and t-tests were unpaired. Treatment with low-dose CTZ (4.5 μM) or 9cRA (1 μM) increased RLU by >300% in both cell lines (t-test: p<0.0001). Combination treatment of low-dosages further augmented RLU activity by approximately two-fold when compared to individual treatments, and by a >500% increase when compared to controls for both cell lines (t-test: p<0.0001). Difference between control, individual, and combination treatments at these concentrations were significant for CA 9-22 cells [F(3,32)=351.7, p<0.0001)] and NA cells [F(3,29)=232.2, p<0.0001)]. These experiments were repeated multiple times with similar results.*

Oil red O. CA 9-22 cells were cultured in 12-well plates at 10×10^4 cells/well in complete RPMI media and allowed to attach overnight. Cells were allowed to grow to 50-60% confluence and experimental groups were then treated with CTZ alone, CTZ plus 9cRA, 9cRA alone, or vehicle solvent, DMSO, for 72-96 h. Cell cultures were incubated at 37˚C in 5% CO_2. Oil red O solution (0.2% in isopropyl alcohol) was then applied for 15 minutes; cells were then rinsed twice with 50% isopropyl alcohol and finally with deionized water. Digital microphotographs were obtained using a SPOT Junior camera with SPOT Basic imaging software (SPOT Imaging, Sterling Heights, MI, USA). During analysis, all images were converted to 16-bit files in ImageJ software and standard background subtraction was applied. Standardized threshold techniques were used in order to identify vacuoles. Images were converted to binary masks and subsequently analyzed. The results were split into quadrants and independently assessed for vacuoles per cell. A minimum of 10 cells were included in the analyses of each test group.

Statistical analysis. Data are presented as mean±standard deviation. Graph Pad Prism 8 was utilized for all statistical analyses. Unpaired two-tailed *t*-test was used when comparing two treatment groups. ANOVA and other tests with post corrections are listed in the results section and figure legends as indicated. *p*-Values <0.05 were considered significant.

Results

Luciferase reporter gene assays. We first examined functional activation of PPARγ by 9-*cis*-retinoic acid (9cRA) and/or ciglitazone (CTZ) in NA and CA 9-22 cell lines, as judged by luciferase reporter gene activity (expressed as RLU, relative luciferase units). Data were analyzed for statistical significance *via* one-way ANOVA analyses with Tukey post-test and unpaired student *t*-tests. With respect to NA cell lines, independent treatments with both low-dose CTZ (4.5 μM) or 9cRA (1 μM) significantly increased PPARγ functional activity by >200% (*t*-test: *p*<0.0001). One-way ANOVA analysis of 9cRA treatments also showed significant dose-dependent differences between control, low (1 μM), and high-dose (10 μM) treatments [F(2,21)=178.1, *p*<0.0001)] (Figure 1A).

Combination 4.5 μM CTZ and 1 μM 9cRA treatment augmented PPARγ DNA binding activation, resulting in an average of >500% increase when compared to control, and an average 2-fold increase compared to treatment only with 4.5 μM CTZ (*t*-test: *p*<0.0001). One-way ANOVA showed significant dose-dependent differences between treatment

groups 4.5 μM CTZ, 4.5 μM CTZ + 1 μM 9cRA, 4.5 μM CTZ + 10 μM 9cRA, and control [F(3,29)=232.2, $p<0.0001$]. Combination treatment with 9 μM CTZ and 1 μM 9cRA also significantly increased PPARγ activation in comparison to treatment only with 9 μM CTZ (*t*-test: $p<0.0001$) (Figure 1A). These data demonstrate that both 9cRA and CTZ can functionally activate PPARγ individually, and the addition of 9cRA to CTZ increases the activation over a given concentration of CTZ alone.

Drug effects on CA 9-22 cells were similar to those in NA cells. Individual treatments with low-dose of CTZ or 9cRA significantly increased PPARγ DNA binding activity by an average of >300% and >200% increases, respectively (*t*-test: $p<0.0001$). One-way ANOVA analysis of the 9cRA effects confirmed statistically significant dose-dependent differences between vehicle control, low, and high-dose treatment conditions [F(2,24)=1,231, $p<0.0001$] (Figure 1B). For both CA 9-22 and NA cells, 9 *μ*M CTZ in combination with 9cRA is associated with marked increased toxicity and cell death, as will be demonstrated in proliferation assays, indicating that lowered luciferase activity is associated with attenuated cellular activity.

Combination of 4.5 μM CTZ and 1 μM 9cRA treatment significantly increased PPARγ activity by an average of >80% compared to individual 4.5 μM CTZ treatment and >600% when compared to control (*t*-test: $p<0.0001$). One-way ANOVA confirmed a statistically significant difference between vehicle control, 4.5 μM CTZ, and 4.5 μM CTZ combined with low and high-dose 9cRA [F(3,32)=351.7, $p<0.0001$] (Figure 1B). Nine μM CTZ increased the rate of PPARγ transcription by >200% when compared to control (*t*-test: $p<0.0001$). DNA binding activity again was augmented by adding 1 μM 9cRA to 9 μM CTZ treatment (*t*-test: $p<0.001$). One-way ANOVA analysis of these two experimental conditions in comparison to control indicated statistically significant differences [F(2,24)=268.2, $p<0.0001$] (Figure 1B). Similar findings in our two oral cancer cell lines demonstrated that retinoids, in addition to thiazolidinediones, augmented PPARγ functional activation in oral cancer *in vitro*.

EMSA binding reactions and shift assays. To confirm the DNA binding reporter gene experiments, nuclear extracts were used for the identification of PPARγ heterodimer binding partners in CA 9-22 cells treated with 9cRA, or 9cRA with ciglitazone. In control cells, we found PPARγ heterodimer binding partners bind to a PPARγ consensus sequence at baseline (Figure 2, lane 2). Next, when cells were incubated with PPARγ, RXRα, RXR β or RXRγ antibodies, PPARγ antibody supershifted the binding complex (Figure 2, lane 5), and a band corresponding to RXRα nuclear protein was found in this complex as well (Figure 2, lane 3). When cells were also treated with 9cRA, we found intensification of the PPARγ shift complex (Figure 2, lane 6). We also found
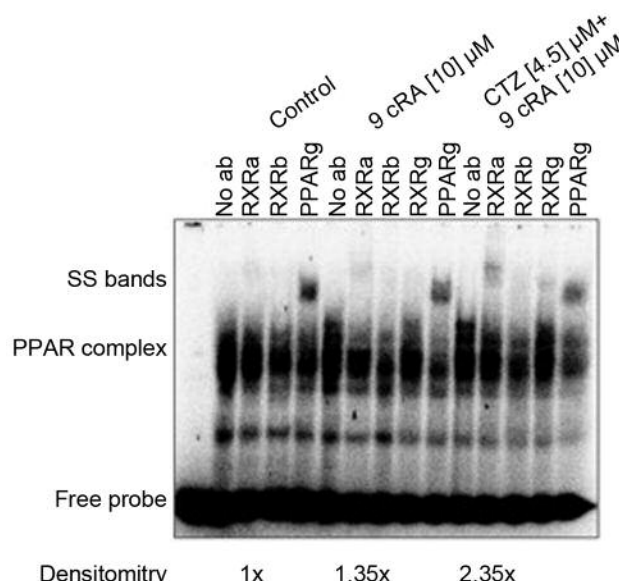

Figure 2. *PPARγ and RXRα EMSA supershift analysis of CA 9-22 cells after ciglitazone and 9-cis-retinoic acid treatment. Lane 1 is the control containing only the free probe. Lane 2 (no ab lane) demonstrates that PPARγ and RXRα bind to PPARγ consensus sequence at baseline. RXRα (Lane 3), RXRβ (Lane 4), and PPARγ (Lane 5) antibodies were preincubated with cells. RXRγ was not used in the control due to lack of available space on the gel. Antibody pre-incubation treatments were performed, including RXRγ (which is not shown on the gel), on cells treated with 10 μM 9cRA (Lanes 6-10) as well as 4.5 μM CTZ + 10 μM 9cRA treatment (Lanes 11-15). The PPAR complex is representative of the RXRα-PPARγ heterodimer formation. SS bands are representative of supershift bands where an antibody is bound to the PPARγ-RXRα heterodimer. There was a PPARγ supershift for all treatment conditions (Lanes 5,10,15). A faint supershift band is found in RXRα antibody treatments (Lanes 3,7,12). There is a faint supershift band in Lane 14 associated with RXRγ antibodies. PPAR complex formation in the control was standardized to the value of 1. Treatment with 10 μM 9cRA resulted in a 35% increase in intensification of the PPARγ shift complex (Lane 6) while the combination treatment resulted in a 135% increase in comparison to control (Lane 11). This experiment was repeated with similar results.*

increasing density of the RXRα band (Figure 2, lane 7) as well as a PPARγ supershift complex (Figure 2, lane 10). Treatment with both ciglitazone and 9cRA resulted in a further intensification of the PPARγ band (Figure 2, lane 11). Additionally, there was intensification of the RXRα supershift band (Figure 2, lane 12). We observed a faint band in the lane containing antibody to RXRγ (Figure 2, lane 14), as well as a supershift complex when using the PPARγ antibody (Figure 2, lane 15). Densitometry performed on the PPAR complex bands without antibodies revealed a greater than 2.35 times increase in the formation of PPARγ-RXRα heterodimer transcription factor in cells treated with both CTZ (4.5 μM) and 9cRA (10 μM) over that of controls. Functional heterodimer formation was 1.5 times greater in CA 9-22 cells treated with 9cRA (10 μM) compared to control. These data
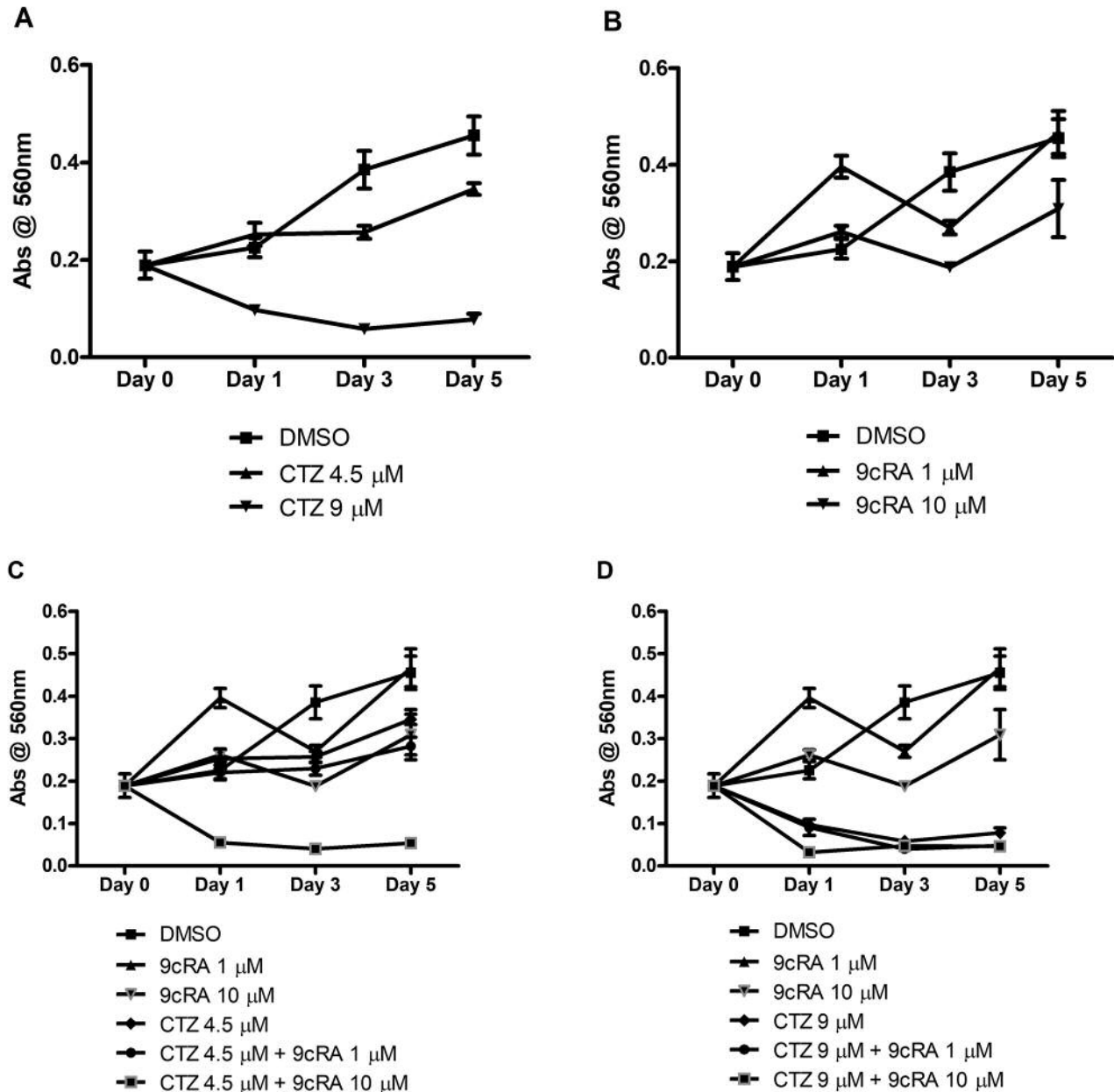
Figure 3. *Cell proliferation of CA 9-22 cells. Individual (A, B) treatments, as well as low-dose (C) and high-dose (D) CTZ combination treatments were analyzed for absorbance at 560 nm. Each data point is the average of 6 replicates. A two-way ANOVA with Bonferroni post-test was employed during analysis. Increasing concentrations resulted in decreased proliferation during treatment with CTZ alone (panel A, p<0.00001) or 9cRA alone (panel B, p<0.001). Combination treatment (panels C, D) with CTZ and 9cRA significantly decreased proliferation (p<0.0001) compared to vehicle control. Furthermore, increasing the dosage of one therapeutic agent further attenuated proliferation (p<0.0001) in comparison to low-dose combination treatment (panels C, D). This experiment was repeated multiple times with similar results.*

demonstrate that both CTZ and 9cRA result in the formation of a nuclear PPARγ-RXRα heterodimer supershift complex. The appearance of a RXRγ element after treatment is intriguing as this transcription factor protein has not been exploited for cancer prevention or treatment. In total, the

EMSA binding assays support the increased functional activity demonstrated in the luciferase experiments. These data are consistent with the reporter gene findings in Figure 1 and provide confirmation that the presumed functional heterodimer is primarily PPARγ-RXRα.
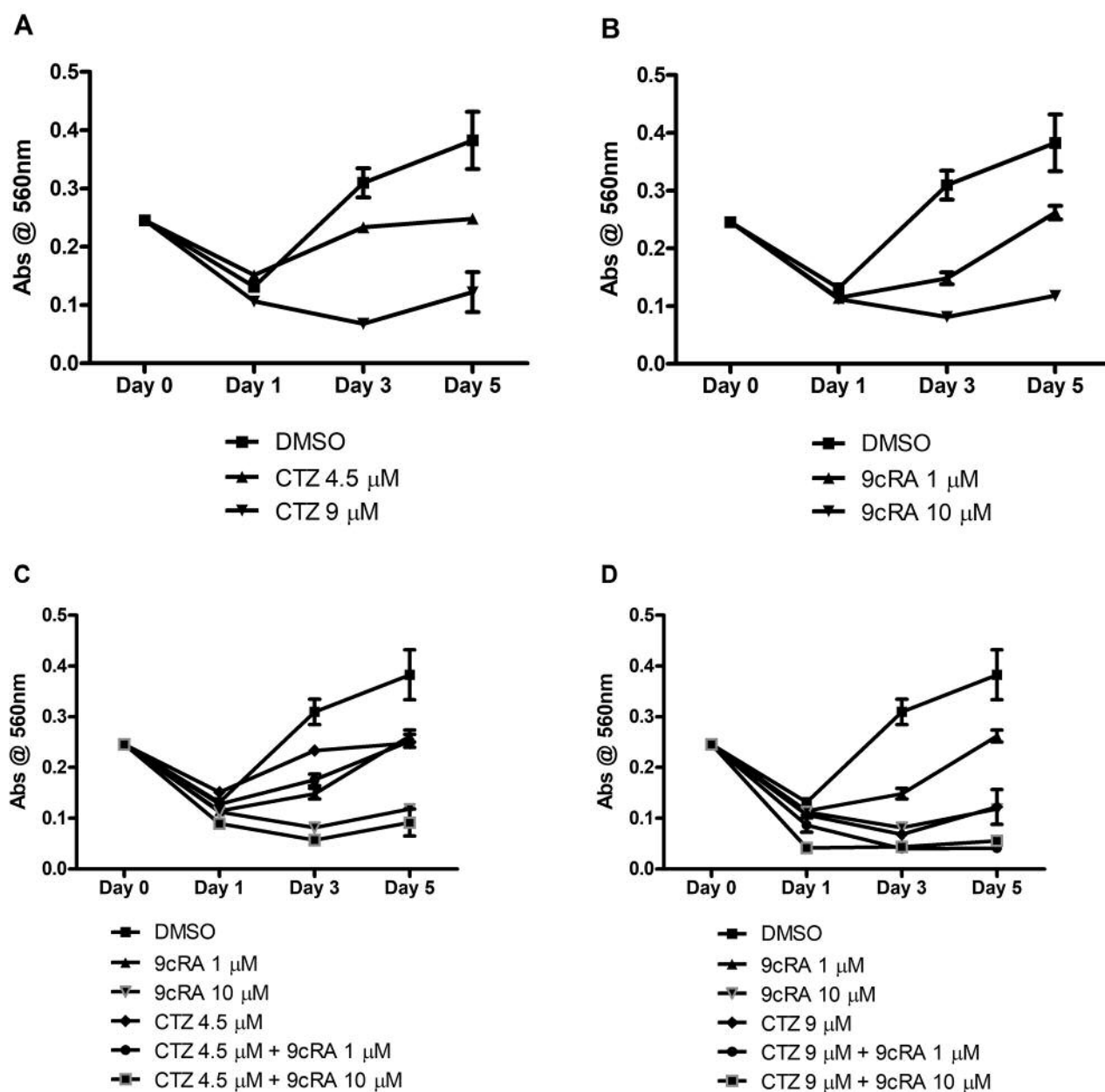
Figure 4. *Cell proliferation of NA cells. Individual (A, B) treatments, as well as low-dose (C) and high-dose (D) CTZ combination treatments were analyzed for absorbance at 560 nm. Each data point is the average of 6 replicates. A two-way ANOVA with Bonferroni post-test was employed during analysis. Treatment with CTZ alone (panel A) or 9cRA alone (panel B) significantly decreased proliferation in comparison to control for all concentrations (p<0.0001). High-dose of CTZ and 9cRA further attenuated proliferation in comparison to low-dose treatments (p<0.0001). Combination treatment (panels C, D) significantly attenuated proliferation for all dosing combinations (p<0.0001). Increasing the concentration of one therapeutic agent further decreased proliferation in comparison to low-dose combination treatments (p<0.0001). This experiment was repeated multiple times with similar results.*

MTT cell proliferation assay. Next, to examine a possible anti-carcinogenic effect associated with PPARγ activation, we analyzed cell proliferation *via* the MTT assay. A two-way ANOVA with a Bonferroni post-test was employed to analyze proliferation data in cell lines at day 5 after

treatment with the agents, alone or in combination. In CA 9-22 cells, treatment with 4.5 μM CTZ significantly decreased proliferation in comparison to control (*t*-test: *p*<0.025) (Figure 3A). There was a greater effect with higher doses of CTZ (9 μM) (*p*<0.0001) and 10 μM 9-cRA (*p*<0.01) with
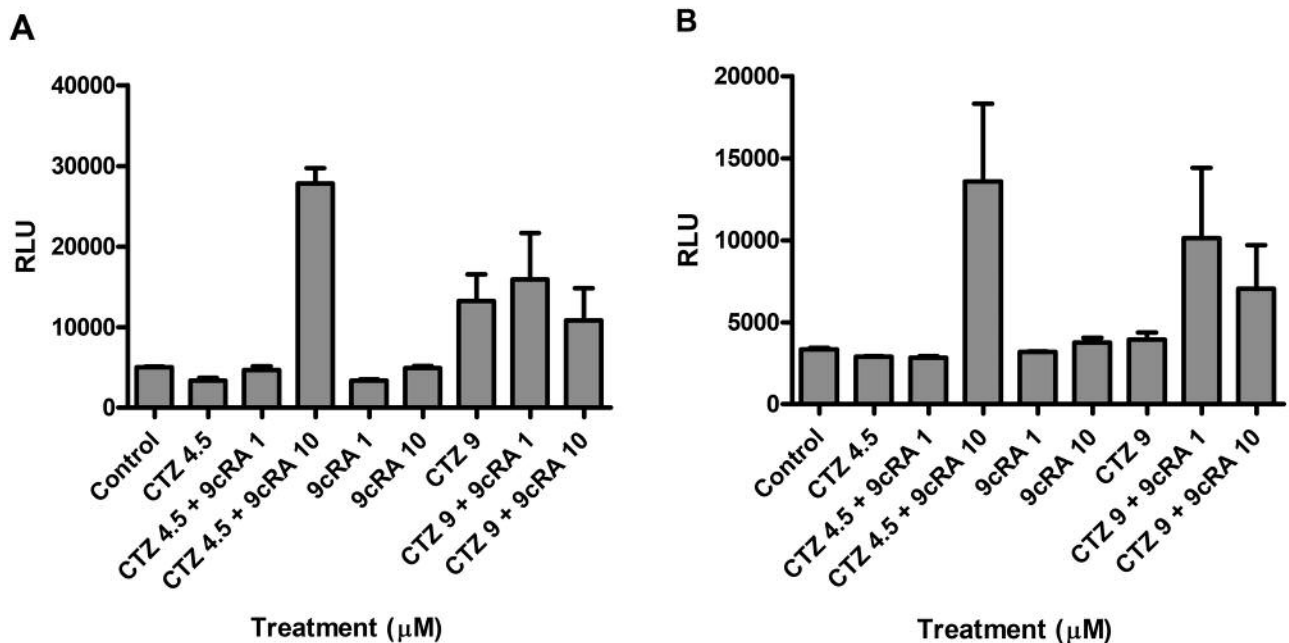
Figure 5. *Apoptosis was analyzed via caspase 3/7 cleavage activity in NA and CA 9-22 cells treated separately with each drug or in combination. A) CA 9-22 cells and B) NA cells. Data is the average of 3 replicates from 3 experiments. Note that combination treatments resulted in high caspase activation for the 4.5 μM concentration of ciglitazone (CTZ). These experiments were repeated multiple times with similar results.*

both significantly attenuating proliferation (Figure 3A, B). There was a dose-dependent decrease in proliferation of CA 9-22 cells treated independently with CTZ ($p<0.0001$) or 9cRA ($p<0.001$) (Figure 3A, B).

Regarding combination therapy, CTZ and 9cRA concentrations significantly decreased proliferation ($p<0.0001$) compared to vehicle control. Proliferation was further attenuated when increasing the dosage of one therapeutic agent while holding the other at a low dose ($p<0.0001$) in comparison to low-dose combination treatment. This was true for both CTZ and 9cRA (Figure 3C, D). There was no added benefit in increasing both concentrations in comparison with increasing the concentration of only one therapeutic agent. For example, 4.5 μM CTZ + 10 μM 9cRA treatment attenuated proliferation significantly more than 4.5 μM CTZ + 1 μM 9cRA ($p<0.0001$), however, yielded relatively the same effect as 9 μM CTZ + 10 μM 9cRA. However, one must also note that at 9uM CTZ that there is essentially a maximal effect of the the agent on MTT with little capacity to augment the effect further with 9cRA.

With respect to NA cell lines, all individual treatments, both low and high-dose, significantly decreased proliferation in comparison to control ($p<0.0001$). High-dose conditions of both CTZ and 9cRA further attenuated proliferation in comparison to low-dose treatments, demonstrating a dose-dependent response to both individual treatments ($p<0.0001$) (Figure 4A, B).

Combination therapy significantly decreased proliferation for all dose concentration combinations in comparison to control ($p<0.0001$). Similar to CA 9-22 cells, increasing the concentration of one agent further decreased proliferation in comparison to low-dose combination treatment ($p<0.0001$). This was true for both CTZ and 9cRA. Again, there was no statistically significant added benefit in increasing the concentrations of both drugs in comparison to increasing the concentration of a single agent (Figure 4C, D). These results indicate that individual targeting of RXRα or PPARγ proteins activates pathways associated with decreased cell proliferation. Combination therapy targeting both principal components of the PPARγ/RXRα heterodimer further potentiated this effect.

Caspase 3/7 cleavage activity assay. We next examined apoptosis, as judged by caspase 3/7 activity, in a series of substrate cleavage luciferase experiments. This was examined in both cell lines after treatment with CTZ or CTZ + 9cRA. In CA 9-22 cells we did not observe significant increases in caspase 3/7 activity following treatment with 4.5 μM CTZ alone or with 4.5 μM CTZ + 1 μM 9cRA. However, treatment with 4.5 μM CTZ + 10 μM 9cRA resulted in a 6-fold increase in caspase 3/7 activation [$F_{(3,8)}=140.7$, $p<0.0001$; ANOVA]. Further, treatment with 9 μM CTZ alone, 9 μM CTZ + 1 μM 9cRA, or 9 μM CTZ + 10 μM 9cRA resulted in a 2-3 fold increases in caspase 3/7 cleavage.

However, this was not statistically significant ($p=0.067$). Similar results were obtained using NA cells. We found that treatment with 4.5 μM CTZ + 10 μM 9cRA resulted in a 5 fold increase in caspase 3/7 activation [$F(3,8)=4.91$, $p<0.033$). Treatment with 4.5 μM CTZ alone or 4.5 μM CTZ + 1 μM 9cRA did not demonstrate significant increases. Furthermore, treatment with 9 μM CTZ, 9 μM CTZ + 1 μM 9cRA, or 9 μM CTZ + 10 μM 9cRA resulted in 2-2.5 fold increases in caspase 3/7 cleavage that was not statistically significant. Over a series of several experiments, we found that addition of 1 or 10 μM 9cRA to 4.5 μM CTZ did not produce significant activation of caspase 3/7 (Figure 5). These effects occur at the same agent concentrations where toxicity was also observed *via* MTT assay (Figure 3, 4). In these experimental concentrations, these data demonstrated that CTZ + 9cRA could stimulate apoptosis in oral cancer cell lines, as judged by caspase 3/7 activation.

Oil red O. PPARγ activation is associated with differentiation in adipose tissue. We were interested in examining whether this would be the case in squamous cancer cells. We assessed whether CTZ and/or 9cRA could be associated with an adipose tissue transdifferentiation response in the squamous cell lines. We evaluated the number of lipid vacuoles present in CA 9-22 cells, as judged by counting oil red O positive droplets after treatment with CTZ and 9cRA. Both, treatment with 1 μM 9cRA and combination of 1 μM 9cRA with 9 μM CTZ resulted in an average of 10 and 17-fold increase in vacuoles/cell, respectively, in comparison to the control (*t*-test: $p<0.01$, $p<0.0001$). Treatment with 9 μM CTZ also resulted in an average of 7-fold increase when compared to control (*t*-test: $p<0.03$) (Figure 6). These data suggest we can partially elicit a lineage reprogramming event to a more adipose-like phenotype by PPARγ activation.

Discussion

The 20-50% survival rate of stage III and IV head and neck squamous cell carcinoma has not significantly improved in the last three decades, revealing the need for innovative treatment options. Our prior research has unveiled that head and neck squamous cell carcinoma tumors had decreased expression of PPARγ, exhibiting a paucity of the function of the heterodimer PPARγ/RXRα transcription factor (13, 20). Exploiting mechanisms to enhance PPARγ expression and activation may reveal anti-cancer activities including decreased proliferation, increased adipocyte differentiation, and apoptosis. Furthermore, using two separate drug classes which are agonists for each principal component of the aforementioned heterodimer (retinoids and thiazolidinediones) may be a way to maximize the efficacy of this mechanism. Prior and current evidence in this manuscript reveals that an activated PPARγ axis is associated with multiple downstream anti-carcinogenic
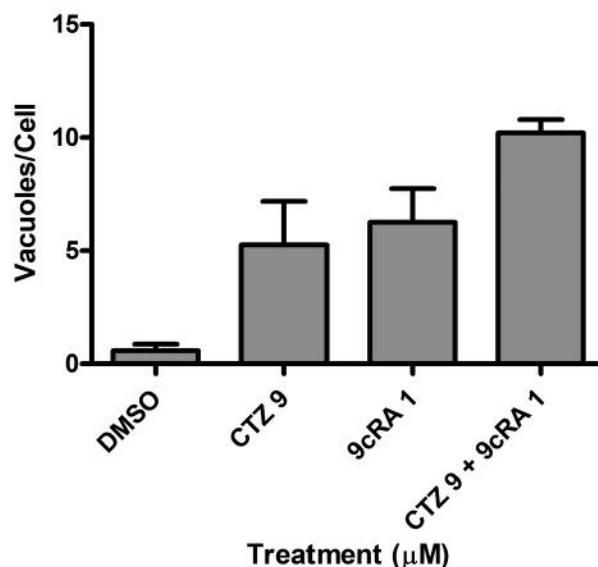

Figure 6. *Oil red O staining of CA 9-22 cell lines. Positive staining for intracellular lipid uptake was indicated by red deposits. Units standardized to vacuoles/cell. A Mann-Whitney U test was employed for 9 μM CTZ to correct for a non-parametric distribution due to outliers above the group mean (p<0.03). One-way ANOVA analysis with Tukey post-test confirmed a statistically significant difference between all treatment conditions [(F(3,12)=9.825, p<0.0015)]. More Oil red O was observed in treated cells than controls in several experiments with similar treatment conditions.*

effects. We showed that two oral cancer cell lines treated with ciglitazone and 9-*cis*-retinoic acid target and activate this axis. Furthermore, functional activation of PPARγ was augmented when these therapeutic agents were used in combination.

Biologically relevant concentrations of 9-*cis*-retinoic acid and ciglitazone were able to independently up-regulate PPARγ-DNA functional activity, as demonstrated *via* reporter gene analysis, by more than 300% in both NA and CA 9-22 cell lines compared to controls, with a dose-dependent response regarding treatment with 9cRA concentrations. EMSA assays confirmed increased PPARγ activation was associated with increased PPARγ/RXRα heterodimer DNA binding in both individual and combined treatment conditions. Combination therapy demonstrated a statistically significant increased effect on PPARγ functional activation in both NA and CA 9-22 cell lines. Low dose 9cRA/CTZ combination treatment was able to increase PPARγ activation approximately two-fold when compared to independent low dose treatments. The favorable activation of the PPARγ-RXRα by both agent classes indicates that titration of each drug class could be clinically exploited to maximize PPARγ activation, eliciting downstream anti-carcinogenic effects while mitigating the toxic effects associated with each drug class.

Cell proliferation assays consistently showed decreased cell proliferation in both cell lines when treated with a ciglitazone and/or 9-*cis*-retinoic acid. In both cell lines there was a dose-depended response to each agent separately (Figures 3 and 4). An additive, augmented effect was observed with the combination treatment over individual treatments. For example, when the ciglitazone concentration was held constant, a 10-fold decrease in 9-*cis*-retinoic acid still retained a comparable ability in reducing cancer cell proliferation. Further, this observation was supported by the apoptosis experiments (Caspase 3/7 activity, Figure 6). The highly favorable decreases in cell proliferation also supported the concept that titration of each drug class could be a highly pragmatic approach for treatment.

Analysis of oil red O staining in CA 9-22 cells corresponded with luciferase data and previous research in that both ciglitazone and 9-*cis*-retinoic acid induced lipid vacuole accumulation through the activation of the PPARγ axis. This would be an expected observation in adipose tissue, however, it is an interesting finding in squamous cancer cells. Furthermore, when therapies were combined, vacuoles/cell increased 17-fold in comparison to baseline (Figure 6). These results provide an additional set of potential pharmacodynamic markers if thiazolidinediones and retinoids were to be used clinically in combination for head and neck squamous carcinoma therapy. Increased vacuole accumulation also correlated with decreased malignant cellular proliferation, aligning with results of previous studies indicating that increased PPARγ activation could result in direct differentiation of malignant cells into a non-malignant, terminal phenotype (12, 17).

Another observation during this project was the increased toxicity when ciglitazone was used at concentrations of 9 μM or higher in combination with 9cRA. At 24 h, there was substantial cell death. This effect was also observed at longer periods of treatment, especially at low serum concentrations. This finding was supported by our observation of actual decreases in MTT below baseline values as the incubation period increases beyond 24 h (data not presented here). These effects are important for clinical cancer treatment; apoptosis, cytotoxicity, and zero proliferation are important for effective cancer treatment. Further research is required to examine if these agents have other (undiscovered) intriguing effects (*e.g.* autophagy).

Combination treatment of tumor cell lines CA 9-22 and NA with PPARγ and RXRα agonists stimulated an increase in PPARγ expression in a dose-dependent fashion. Functional activation of the PPARγ axis resulted in downstream anti-carcinogenic effects including decreased proliferation, increased apoptosis, and up-regulation of adipocyte differentiation markers. These results further substantiate existing evidence from similar cancers, which showed peroxisome proliferator-activated receptor targeting is a viable option for the treatment and/or prevention of carcinogenic growths. Furthermore, the additive/superadditive effects of the combination of these agent classes could allow minimization of toxicity while preserving the desired treatment properties. Given this, as well as the fact that the thiazolidinedione family members and 9-*cis*-retinoic acid are FDA approved for other clinical indications, makes them a feasible, practical combination strategy for treating head and neck squamous cell carcinoma, a disease in need for new therapies.

Conflicts of Interest

The Authors declare they have no conflicts of interest regarding this study.

Authors' Contributions

RS and FO designed the study. RS, RR, and BW carried out all experiments and drafted the manuscript. BW and FO supervised all experiments performed. SB, RR, BW, and FO participated in the data interpretation. SB, BW, and FO prepared the final manuscript. As the principal investigator, FO supervised the study. All Authors read and approved the final manuscript.

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