Biological Evaluation of Novel Rexinoids as a Therapeutic Agent for Cancer and Alzheimer's Disease

by

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ABSTRACT

Bexarotene is a Food and Drug administration (FDA)-approved therapeutic used in the treatment of cutaneous T-cell lymphoma (CTCL). However, bexarotene therapy causes significant side effects like hyperlipidemia and hypothyroidism due to crossover activity with retinoic acid receptor (RAR), thyroid hormone receptor (TR), and liver X receptor (LXR) signaling, respectively. More recently bexarotene has shown promise to reverse neurodegeneration, improve cognition and decrease levels of amyloid-β in transgenic mice expressing familial Alzheimer's disease (AD) mutations. Bexarotene is a high affinity ligand for the retinoid X receptor (RXR) that heterodimerizes with the liver-X- receptors (LXR) and with peroxisome proliferator-activated receptor-gamma (PPARϒ) to control cholesterol efflux, inflammation, and transcriptionally upregulates the production of apolipoprotein (ApoE) in the brain. Enhanced ApoE expression may promote clearance of soluble Aβ peptides from the brain and reduce Aβ plaques, thus resolving both amyloid pathology and cognitive deficits. The present study assessed the potential of bexarotene and a group of 62 novel rexinoids to bind and activate RXR using a series of biological assays and screening methods, including: 1) a mammalian two-hybrid system (M2H) and an 2) Retinoid X Receptor response element (RXRE)-mediated reporter assays in cultured human cells. Moreover, Liver X Receptor response element (LXRE)-mediated luciferase assays were performed to analyze the ability of the novel analogs to activate LXRE - directed transcription, and to induce ApoE messenger ribonucleic acid (mRNA) in U87 glial cells. Furthermore, the most potent analogs were

analyzed via quantitative polymerase chain reaction (qPCR) to determine efficacy in modulating expression of two critical tumor suppressor genes, activating transcription factor 3 (ATF3) and early growth response 3 (EGR3). Results from these multiple assays indicate that the panel of RXR ligands contains compounds with a range of activities, with some analogs capable of binding to RXR with higher affinity than others, and in some cases upregulating ApoE expression to a greater extent than bexarotene. The data suggests that minor modifications to the bexarotene core chemical structure may yield novel analogs possessing an equal or greater capacity to activate RXR and may be useful as therapeutic agents against CTCL and Alzheimer's disease.

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I could not have undertaken this journey without the support of my lab mates, Flavio, Sarah, Zhela, Jennifer, Mobin, Michael and Jose. Our time together will always be one of my most special memories. I will miss you guys.

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LIST OF ABBREVIATIONS

9-*cis*-RA	9-*cis*-retinoic acid
AD	Alzheimer's Disease
AF-1	Activation Function-1
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
ATF3	Activating Transcription Factor 3
ATP	Adenosine Triphosphate
ATRA	All-*trans*-Retinoic Acid
B-RAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B1
c-MYC	Cellular Master Regulator of Cell Cycle Entry and Proliferative
CTCL	Cutaneous T- Cell Lymphoma
DBD	DNA-Binding Domain
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxy Ribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
EGR3	Early Growth Response 3
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HEK-293	Human Embryonic Kidney 293 Cells
HRE	Hormone Responsive Element

Hut78	Human Cutaneous T-Lymphocyte
K-RAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LBD	Ligand-Binding Domain
LXR	Liver X Receptor
LXRE	Liver X Response Element
M2H	Mammalian 2 Hybrid
MF	Mycosis Fungoides
mTORC	Mammalian Target of Rapamycin Complex
NLS	Nuclear Localization Sequence
NMDA	N-methyl-D-aspartate
NR	Nuclear Receptors
NTD	N-Terminal Domain
PEI	Polyethylenimine
PI3K	Phosphoinositide 3-Kinase
PPAR	Peroxisome Proliferator-Activated Receptor
PSEN1	Presenilin-1
PSEN2	Presenilin-2
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Receptor Element
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor

RXRE	Retinoid X Receptor Element
SS	Sézary Syndrome
STR	Short Tandem Repeat
TGF-α	Transforming Growth Factor Alpha
TR	Thyroid Receptor
U87	Human Glioblastoma Cells
VDR	Vitamin D Receptor
Wnt	Wingless/Integrated

INTRODUCTION TO THE THESIS

The following experiments and data analysis were performed by me.

Generation 6, 7, 9, 10 and 11 LXRE assays

Generation 9 and 10 RXRE assays

Generation 9, 10 and 11 M2H assays

Generation 9 and 10 RARE assays

Generation 9, 10 and 11 ApoE qPCR

The following experiments were performed by Ankedo Warda.

Generation 6 and 7 M2H and RXRE assays.

The following experiments were performed by Michael Sausedo under my guidance.

Generation 8 RARE assays

The following experiments were performed by Zhela Sabir under my guidance.

Generation 6 and 7 RARE assays

The following experiments were performed by Sanchita Mallick and some data was re-analyzed by me.

Generation 8 M2H, RXRE and LXRE assays

Generation 8 qPCR

The following experiments were performed by me, Sarah Livingston, and Michael Sausedo.

Generation 6 and 7 anti-cancer genes qPCR.

CHAPTER 1

INTRODUCTION

The Nuclear Receptor Superfamily

Nuclear receptors belong to a super-family of ligand-modulated transcription factors that are activated by steroid hormones, such as estrogen and progesterone, and various other lipid-soluble ligands, such as retinoic acid, oxysterols, and thyroid hormone (Mangelsdorf et al., 1995). These ligands possess the ability to cross the plasma membrane and foster interaction with nuclear receptors inside the cell, bypassing the need to act via cell surface receptors. Following ligand binding, activated nuclear receptors directly regulate transcription of genes that control a wide spectrum of biological processes, such as cell proliferation, development, metabolism, reproduction, and apoptosis. Nuclear receptors predominantly function as transcription factors however, a few nuclear receptors can also regulate cellular functions within the cytoplasm (Sever and Glass, 2013).

Structure

All nuclear receptor superfamily members contain four to five common domains labeled A–F (Fig.1). Domains A and B constitute the highly variable (both in length and sequence) amino/N-terminal domain (NTD). This domain also contains the first of two transactivation domains (activation function-1, AF-1). Adjacent to the A/B domain is the highly conserved C domain distinguished by a central conserved DNA-binding domain

(DBD) that directs the receptor to specific DNA sequences known as hormone response elements. The nuclear receptors differ from other DNA-binding proteins due to the presence of two highly conserved zinc fingers in the DBD that act as a hook and allow binding to chromatin within the nucleus (Porter et al. 2019). The D domain or 'hinge' region is a flexible region that generally contains the main nuclear localization sequence (NLS). The carboxy (C)-terminal half of the receptor is composed of the well conserved ligand-binding domain (LBD) which contributes towards hormone receptor recognition and guarantees both specificity and selectivity of the physiologic response. Thus, the LBD acts as a molecular switch that shifts the receptor to a transcriptionally active state upon ligand binding (Mangelsdorf et al., 1995). This region also contains the second transactivation domain (AF-2). Lastly, some nuclear receptors also possess a highly variable F domain that often has unknown functions (Frigo et al., 2021).

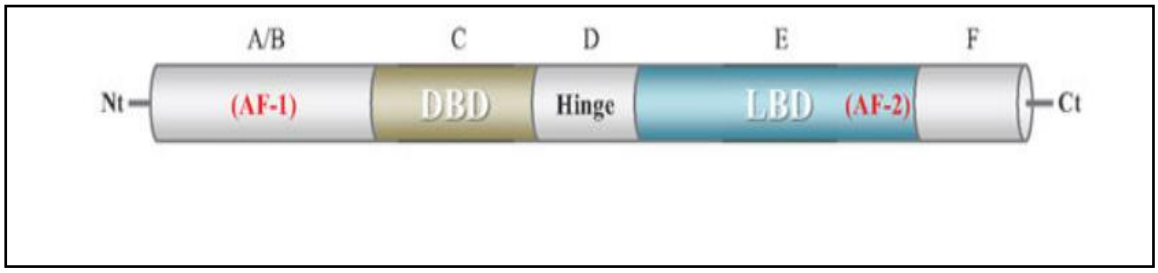

Figure 1: General Nuclear Receptor Structure.

NRs consist of six domains, N-terminal activation function domain, a central DNA binding domain, and a C-terminal ligand binding domain that carries out ligand-dependent transcriptional function (Grimaldi et al., 2015).

Classification

The nuclear receptor super-family consists of over 48 members (Weikum et al., 2018). Based on their dimerization, DNA-binding characteristics, and ligand binding the nuclear receptor super– family can be grouped into four classes (Fig. 2). Although there are some important structural and functional differences between the different classes, some pivotal structural components are preserved which are permissive to their respective functions. Class I receptors encompass the known steroid hormone receptors which function as ligand induced homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors form heterodimers with RXR and bind to direct repeats whilst some may bind to symmetrical repeats. The Class II receptor group includes all other known ligand-dependent receptors excluding the steroid hormones. Class III receptors predominantly bind to direct repeats as homodimers. Class IV receptors primarily bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories (Mangelsdorf et al., 1995).

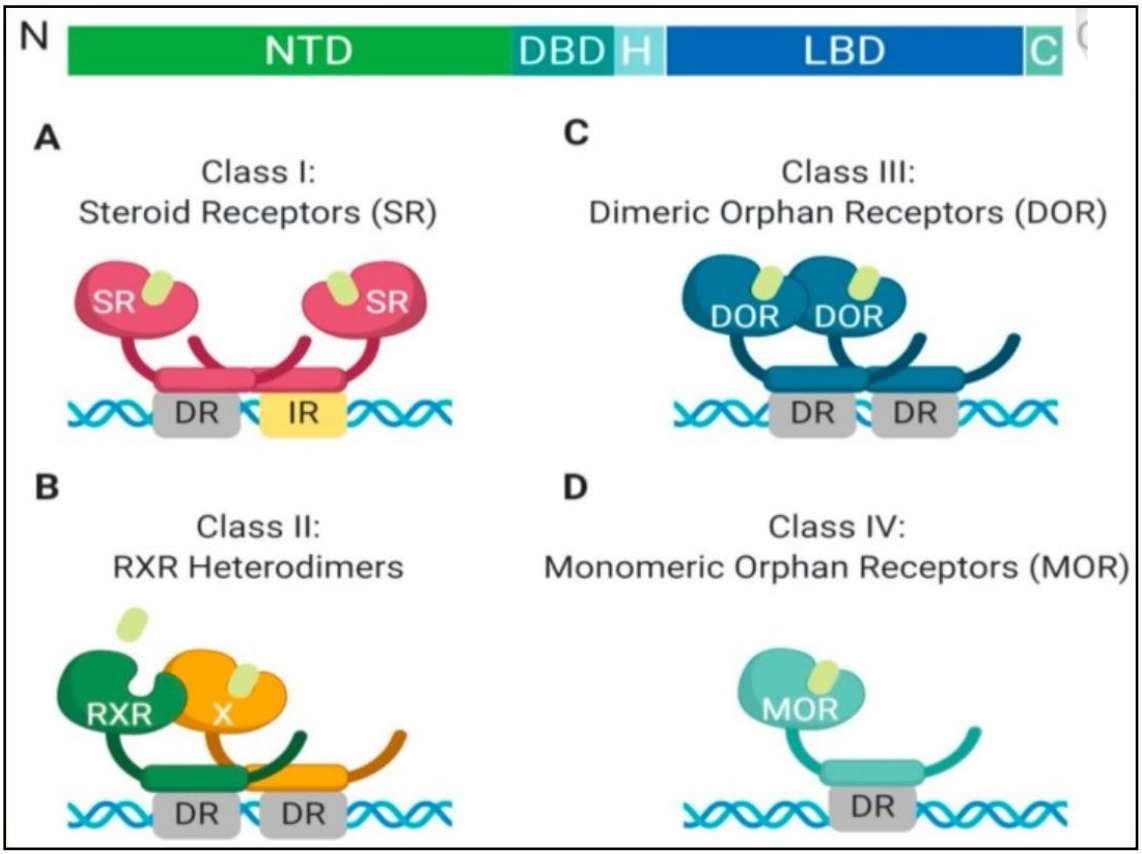

Figure 2*:* Schematic Diagram Of The Nuclear Receptor Superfamily. Depicted are the four classes (A-D) of the nuclear receptor superfamily which are classified based on dimerization (homo, hetero, or mono), DNA binding (direct repeat or inverted repeat), and ligand specificity (essential or non-essential). Class I, Steroid Receptor are also known as nuclear hormone receptors; Class II, RXR Heterodimers; Class III, Dimeric Orphan Receptors; Class IV, Monomeric Orphan Receptors. Abbreviations: NTD, N-terminal domain; DBD, DNA-binding domain; H, Hinge region; LBD, Ligand-binding domain; C, Variable C-terminus; DR, Direct Repeat; IR, Inverted Repeat (Porter et al., 2019).

The Human Retinoid X Receptors

The human retinoid X receptors consist of three identified isoforms (α, β, γ) (Mangelsdorf and Evans 1994; Leid et al.,1992) with one or more of the isoforms exhibiting expression in every human tissue type where the receptor regulates—

sometimes in partnership with other nuclear receptors—gene transcription, often stimulated by receptor-specific molecular signaling. The RXRs display a remarkable versatility unknown among other nuclear receptors (NRs) making up a transcriptional modulator superfamily because they join with many of the NRs to create heterodimers that actively modulate the pathways central to cell differentiation, metabolism, proliferation, and migration. Some of the critical receptor pathways where RXR participates as an essential component to realize functional responses include the liver X receptor (LXR), the thyroid hormone receptor (TR), the peroxisome proliferator-activated receptor (PPAR), the vitamin D receptor (VDR), and the retinoic acid receptor (RAR), to name a few. All NRs control gene expression primarily by regulating transcription, and usually in response to the presence of an associated receptor ligand and their obligate partnering receptor. Receptor ligands, often endogenous molecules, bind to the receptor's ligand-binding domain (LBD), which, in turn, compels the receptor to adopt a conformation that can then dimerize with an additional receptor, recruit co-factors, and ultimately bind with high affinity to a specific hormone responsive element (HRE) that the receptor recognizes in DNA. Increasingly, HREs are being identified considerably up- or downstream from their moderated genes; however, many HREs have also been identified close to or within the promoter region of the regulated genes. Initially, TRs, VDRs, and RARs were presumed to bind to their HREs as homodimers (Forman et al.,1989), though they were later discovered to associate with RXR as a prerequisite to binding and activating their HREs (Forman et al., 1989). Zhang and colleagues first

reported that 9-*cis*-retinoic acid (9-*cis*-RA)—a naturally occurring isomer of all-*trans*-retinoic acid (ATRA)—is an RXR-specific ligand that functions as an agonist where its binding to RXR compels the formation of RXR homodimers and subsequent association with RXR responsive elements (RXREs) (Zhang et al., 1992). When RXR associates with other NRs as a heterodimer, the heterodimer does not need to possess an RXR-specific ligand in the LBD for RXR. For example, the RXR-VDR heterodimer has been reported to function without a ligand bound to RXR (Thompson et al., 2001). Alternatively, some RXR heterodimers exhibit enhanced activity with RXR-specific ligands (rexinoids) bound to RXRs' LBD, as in the case of the RXR-LXR heterodimer (Svensson et al., 2003). Considering this degree of versatility—the necessity for RXR to partner with several NRs with or without ligands for those NRs to function—RXR has reasonably been termed the master receptor.

Numerous RXR studies, comprising multitudes of rexinoids with different partnering NRs, have distilled two primary RXR heterodimer classifications—the so-called permissive and non-permissive RXR heterodimers (Forman et al., 1995). Only the heteropartner's agonists can activate purely nonpermissive RXR heterodimers, whereas either the heteropartner's agonists or rexinoids can activate permissive RXR heterodimers. The RXR-RAR, RXR-TR, and RXR-VDR heterodimers have all been characterized as non-permissive. In most, but not all conditions, the RXR partnering receptor for the VDR and TR heterodimers is "silent." The RXR-RAR heterodimer, on the other hand, showed enhanced activation by both certain rexinoids and RAR-specific

agonists. Specific RXR agonists, in fact, have shown activation of RXR-RAR in the absence of RAR-specific agonists (Lala et al., 1996). The primary classification of RXR-RAR as nonpermissive has evolved considering these observations to have a more accurate "conditionally nonpermissive" designation. The RXR-LXR, RXR-PPAR, and RXR-FXR heterodimers, alternatively, are all known to be fully permissive.

Both permissive and nonpermissive RXR heterodimers often give rise to pleiotropic effects from exposure to potent rexinoids—the former by stimulating RXR heterodimer pathways and the later by titrating a finite pool of RXR away from participating in the proper formation and functioning of those nonpermissive RXR heterodimers. This potential for pleiotropy has frustrated the clinical development of rexinoids for therapeutic applications. Rexinoids such as 9-*cis*-RA can arrest the functioning of the RXR-VDR (Lemon and Freedman, 1996; MacDonald et al., 1993; Thompson et al., 1998) and RXR-TR (Lehmann et al., 1993) heterodimers.

Nevertheless, the two overarching characteristics concerning the development of rexinoid therapeutics that exert fewer side effects and greater benefits comprise selectivity and potency (Jurutka et al., 2013). Thus, an approach to modify a parent RXR agonist's structure may impact both potency and RXR-heterodimer selectivity, leading to improved pleotropic profiles by generating specific NR modulators.

Cancer

A cancerous tumor is defined as an aggregation of cells proliferating rapidly in comparison to normal tissue, in a rampant fashion and that has invasive and metastatic

properties (Roy & Saikia, 2016). As per the latest data, the mortality caused by cancer has dwindled over the past decade in both males and females, however it is still the second most common cause of mortality and morbidity in the United States next to cardiovascular disease (Siegel et al., 2018; Xu et al., 2016). The major cause of cancer development are mutations in one or more genes regulating the cell cycle. In some cases, the defective gene is inherited while as in other cases, the mutations are caused due to exposure to environmental carcinogens. Typically, in majority of the cancer cases both hereditary and environmental factors play a role, and an accumulation of mutations is a prerequisite for the cancer to develop (Iranzo et al., 2018; Zhu et al., 2015). When the DNA of the cell is damaged due to environmental factors like UV radiation, various repair mechanisms are activated to repair the damage. However, if the repair mechanisms are unable to mend the damage, cell death signals are activated but if there is a disruption in those mechanisms the mutated cells can proliferate (Goldar et al., 2015; Torgovnick & Schumacher, 2015). Resistance to the effects of cell death signals and immortality are the paramount properties of cancerous cells. There are three major classes of programmed cell death: apoptosis, autophagic cell death, and necrosis programmed cell death; all of them determine the fate of the cancer cell in conjunction (Jain et al., 2013). Out of the three pathways, apoptosis has been extensively studied, and research on cancer treatment has focused on mechanisms to induce apoptosis in cancer cells.

The genetic changes that drive cancer affect three major types of genes: Proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes play an important role in normal cell growth and division. However, when these genes are affected by mutations, they can become cancer-causing genes (or oncogenes), allowing unchecked cell growth. Oncogenes encode proteins that stimulate tumorigenesis. Tumor suppressor genes inhibit the development of tumors. p53 is among the most important tumor suppressor genes involved in controlling the cell cycle and induction of apoptosis. K-RAS, B-RAF, and c-MYC are crucial oncogenes (Croce, 2008; M. Zhao et al., 2012). The AKT/PI3K, RAS-ERK, mTORC, and Wnt/B-catenine signaling pathways have a substantial effect on the metabolism of cancerous cells (Martin, 2003; H. Yao et al., 2011). Otto Warburg in the 1920s observed increase glucose uptake by cancer cells compared to normal cells which led to the proposition that there is a remarkable difference in the metabolism of cancerous cells in comparison to normal cells (Warburg et al., 1927). Like normal cells, cancer cells also have a requirement to synthesize macromolecules for growth and proliferation. However, cancer cells are well equipped to adapt to adverse conditions such as hypoxia and nutrient deprivation. Furthermore, ATP production and utilization, as well as the redox balance in cancer cells are highly regulated. Some therapeutic approaches are concentrated towards targeting the peculiar metabolic features of cancer cells (Hornsveld & Dansen, 2016; Phan et al., 2014; Reid & Kong, 2013; Samanta & Semenza, 2018).

Cutaneous T- cell Lymphoma (CTCL)

Skin can be affected by diverse types of T- and B-cell neoplasms, either primarily or secondarily. Primary cutaneous lymphoma are a type of cutaneous T-cell lymphomas (CTCLs) and cutaneous B-cell lymphomas (CBCLs) that occur in the skin with no indication of extracutaneous disease at the time of diagnosis. The integumentary system is the second most common site of extranodal non-Hodgkin lymphoma, after the gastrointestinal tract. Primary cutaneous lymphomas usually demonstrate different clinical behavior and prognosis from histologically similar systemic lymphomas, which may involve the skin secondarily, and therefore require different types of treatment. Almost 75% of primary cutaneous lymphomas are derived from T-cells, of which two-thirds are further characterized as either Mycosis fungoides (MF) or Sézary syndrome (SS).

Classic MF tends to be a more indolent disease which slowly progresses over the course of several years to decades, advancing through various stages including patches, plaques, and tumor (Jawed et al., 2014; Jonak et al., 2021). Patients with MF present with well-defined and pruritic erythematous patches and plaques often distributed in sun-protected areas including the groin, trunk, buttocks, and breasts. These lesions may have varying sizes and levels of desquamation and can ulcerate in advanced disease. Some patients may present with concurrent patches, plaques, and tumors and although tumors often signify later-stage disease, they may also occur *de novo* (Kim et al., 2003). Although rare, MF may also present with hypopigmented lesions in children, adolescents, and

individuals with dark complexion (Cho et al., 2010). In 2003, Kim et al. performed a study analyzing the clinical characteristics of 525 patients with CTCL and found that 67% of patients initially presented with limited or generalized patches and plaques, while 33% presented with tumors or erythroderma at disease onset (Kim et al., 2003).

Although less common than MF, SS is another distinct and more clinically aggressive type of CTCL. Classic SS is characterized by intensely pruritic and generalized skin involvement with erythroderma, lymphadenopathy, and leukemic spread of malignant CD4+ T cells (Kemph et al., 2021; Kohnken et al., 2016). SS often presents *de novo*; however, it may also develop from long-standing MF, known as "SS preceded by MF" or "secondary SS" (Kohnken et al., 2016). Erythrodermic lesions in SS may range from mild erythema to generalized exfoliative dermatitis and fissuring involving the palms and soles (Jawed et al., 2014). Significant exfoliative lesions are often accompanied by electrolyte and protein loss, hypothermia, and alopecia. The cutaneous lesions of SS can be misdiagnosed as allergy, atopic dermatitis, adverse drug reactions, or chronic contact dermatitis.

Although its pathophysiology is quite complex, CTCL is the result of malignant transformation of skin-homing/resident T-cells (Willemze et al., 2005) and may be more simply explained in terms of its "microenvironment" and "macroenvironment." The observation that T-cell costimulatory signals support the growth of malignant T-cells *in vitro* highlights the importance of extrinsic factors present in the tumor microenvironment (McCusker et al., 1997; Yamanaka et al., 2006)

Furthermore, numerous gene expression profiling and immunohistochemistry-based studies have demonstrated the important role of nonmalignant cells, including lymphoma-associated macrophages and dendritic cells, which may be recruited into the tumor microenvironment by tumor-derived chemokines (Schlapbach, C et al., 2010; Vaque, J.P et al., 2014). These nonmalignant cells are then able to promote tumorigenesis via direct mechanisms such as the production of factors involved in tumor cell growth and survival, and indirect mechanisms such as the promotion of angiogenesis and suppression of host anti-tumor immunity (Wilcox, R.A. 2010).Contributing to the significant morbidity and mortality associated with the infectious complications often seen in CTCL is that of widespread impairment of cellular immunity – the tumor "macroenvironment"(Wilcox, R.A. 2017). It is known that roughly 50% of patients with CTCL, especially those with advanced disease, eventually succumb to the sequelae of infection (Axelrod et al., 1992; Epstein et al., 1972; Posner et al., 1981). This phenomenon is due to both quantitative and qualitative defects in immune system functions, including natural killer cells (Bouaziz et al., 2005; Wysocka et al., 2004), dendritic cells (Wysocka et al., 2002), and T-cell mediated immunity (French et al., 2005; Lee et al., 1999; Samimi et al.,2010) amongst other components such as the loss of T-cell repertoire, analogous to that observed in HIV infection, and loss of T-cell receptor diversity (Wilcox et al., 2017;Yawalkar., et al 2003).

In both MF and SS, disease staging according to the World Health Organization-European Organization for Research and Treatment of Cancer classification system

dictates the choice of treatment. Treatment options range from skin-directed therapies (SDTs) to biologic systemic therapies and, of course, chemotherapy. In early stages of disease, patients often respond well to SDTs alone. In more advanced CTCL however, SDTs may be used in combination with systemic therapy. Common SDTs include alkylating agents, phototherapy, photodynamic therapy, and electron beam therapy (Gardner et al., 2009). The selection of systemic therapies for CTCL is broad, and many agents may be used in combination. These agents include cytokines such as IFNα and IFNγ, methotrexate, denileukin diftitox, histone deacetylase inhibitors, monoclonal antibodies, forodesine, and stem cell transplants. Of most relevance to the current study is the use of bexarotene in the treatment of CTCL.

Bexarotene

Bexarotene (Targretin®) (Fig. 3) is an RXR-selective agonist that binds to all three RXR isoforms (RXRα, RXRβ, and RXRγ) with equivalent affinity and activates the receptors with comparable potency. Bexarotene is approved by the FDA and is a universally used therapeutic for treating cutaneous T-cell lymphoma (CTCL). Moreover, it has been used as an off-label drug for treating non-small cell lung cancer and breast cancer. Bexarotene has shown promise in various in vitro studies in preventing and overcoming acquired drug resistance in advanced breast cancer, non-small cell lung cancer and even advanced prostate cancer. Numerous studies have been carried out to ascertain the efficacy of bexarotene for the treatment of breast cancer due to its antitumor activity, with a special focus on its safety. A research study done in 2003 assessed the use of oral bexarotene in

patients with metastatic breast cancer, where the participants were split into three groups – the first two groups received bexarotene alone, while the third group consisted of tamoxifen-resistant patients that received both tamoxifen and bexarotene (Esteva et al., 2003). A total of 148 patients were tested, and an assortment of results were obtained. Out of all the drugs tested, bexarotene demonstrated minimal toxicity. The most reported side effects were hypertriglyceridemia (84%), dry skin (34%), asthenia (30%), and headache (27%) (Esteva et al., 2003). Approximately 20% of subjects reported clinical benefits with bexarotene treatment and it was reported that bexarotene, due to its specific mechanism and minimum toxicity as compared to other chemotherapeutic drugs, could be a better choice for select breast cancer treatments, but was limited in its overall use. An in vitro analysis demonstrated that bexarotene could arrest cancer cell growth via apoptosis, decrease expression of TGF-α and EGFR, induce cellular senescence associated with increased p21 and p16 expressions, and promote G1-phase cell cycle arrest. However, the explicit mechanism of cell proliferation inhibition by bexarotene remains unclear (Kobayashi et al., 2022).

While bexarotene is predominantly RXR-selective and avoids significant RAR-activation, patients treated with bexarotene often experience hypothyroidism, hyperlipidemia, and occasionally cutaneous toxicity. Bexarotene, like 9-*cis*-RA, incites these side effects by disrupting nonpermissive heterodimers—hypothyroidism by RXR-TR disruption—or stimulating the permissive heterodimers—hyperlipidemia via RXR-

LXR activation and cutaneous toxicity from RAR activity at high dose concentrations (Jurutka et al., 2021).

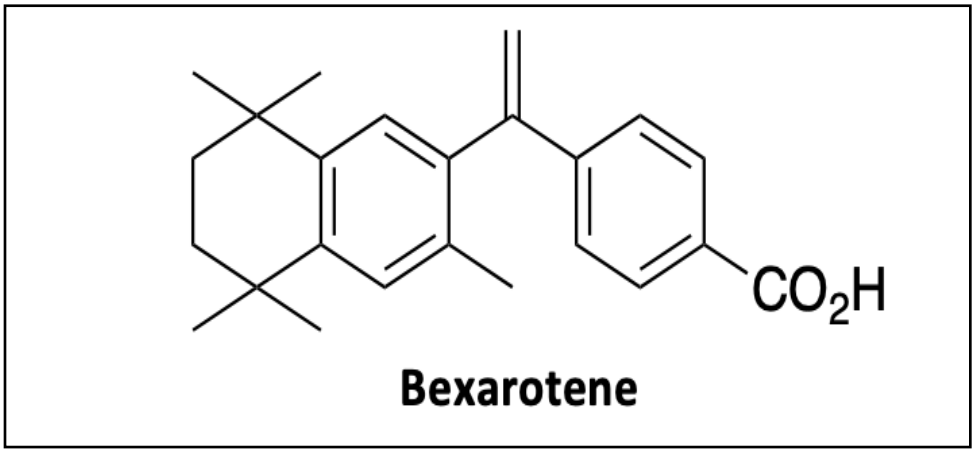

Figure 3: Structure of Bexarotene Molecule

Bexarotene has also shown some promise in neurodegenerative disease models such as Alzheimer's disease (AD) (Cramer et al., 2012). Moreover, several novel rexinoids were recently reported to be equally or more effective at modulating gene expression via LXREs and are thus superior at inducing ApoE, the gene whose enhanced expression is thought to mitigate the pathophysiology associated with Alzheimer's disease (Mallick et al., 2021).

Bexarotene has shown tremendous promise not only as a cancer therapeutic, but also as a potential treatment for neurodegenerative diseases like Alzheimer's disease. Therefore, it becomes essential to further our research in the development of novel rexinoids that are similar to bexarotene in structure and function but at the same time can mitigate the side effects caused by bexarotene therapy.

Alzheimer's Disease

Alzheimer's disease is the most common cause of dementia in the world. The incidence of dementia will increase precipitously in the coming years due to the increase in the aging population. Unfortunately, there is currently no cure for Alzheimer's disease. However, some risk prevention measures in mid-life can probably delay onset of Alzheimer's disease.

In Alzheimer's disease (AD), there is a progressive onset of symptoms associated with the loss of cortical functions. Clinically, there are two phenotypes of the disease, amnestic and non-amnestic form (Mckhann et al., 2011). In the amnestic form, the disease debuts with memory loss for recent events or conversations (episodic memory) and issues with time orientation. Symptoms like reduced understanding, judgment, thinking, and language difficulties develop over time. In the non-amnestic form, the disease begins with behavioral changes, depression, language difficulties, orientation difficulties or visual problems.

Slowly, the patient develops global cognitive impairment, various behavioral and psychological symptoms as well as functional impairment, and most of them go on to develop motor symptoms and autonomic dysfunction. They have a significantly reduced life expectancy compared to their peers (Strand et al., 2018) .

A large number of Alzheimer's disease cases are sporadic, i.e., without a known familial burden (Bertram et al., 2007). The *APOE-ε4* genotype is the most prevalent risk allele for the development of the disease (Kern et al., 2015). Early-onset familial Alzheimer's

disease with mutations in amyloid precursor protein (APP), presenilin-1 (PSEN1) or presenilin-2 (PSEN2) is rare. In addition, greater than 50 genes or gene loci are associated with the disease, but with low penetrance (Scheltens et al., 2016; Lukiw et al., 2020).

The pathological changes in the brain likely begin 10–20 years before the onset of symptoms (Jack et al., 2018). The disease begins with the development and deposition of insoluble amyloid plaques extracellularly. Intracellularly, tau proteins are phosphorylated, resulting in the formation of neurofibrillary tangles. The neurons are damaged, the immune system is activated, and vascular changes occur. The receptors on the nerve synapses are damaged and the production of several neurotransmitter substances is reduced (Lukiw et al., 2020).

AD has no cure and the drugs available to treat AD are only symptom-relieving with no long-term efficacy. The commonly prescribed drugs for symptomatic relief are cholinesterase inhibitors and the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist memantine. Cholinesterase inhibitors are the first choice for the treatment and Memantine is only recommended when cholinesterase inhibitors are not tolerated or have no effect in advanced AD dementia. Combination of the two drugs is only exceptionally recommended in the national professional guidelines for dementia. The three cholinesterase inhibitors rivastigmine, donepezil and galantamine are considered to have similar clinical effects, but somewhat different side effect profiles. They have no documented effect on mild cognitive impairment and do not delay

the development of dementia either (Livingston et al., 2017). Cholinesterase inhibitors stabilize or reduce the changes in cognition, behavior and general (ADL) function (Tan et al., 2014).

The most common side effects are gastrointestinal (diarrhea, nausea and vomiting), but dizziness, headache and sleep difficulties including nightmares are also frequent. As cholinesterase inhibitors can cause bradycardia, care must be taken in people with cardiac conduction disorders or when using other drugs that affect cardiac conduction (e.g., beta-blockers). Memantine inhibits glutamate's effect by acting antagonistically on the NMDA receptor. The indication is moderate to severe AD dementia.

The development of new disease-modifying drugs has been aimed at changing amyloid or tau pathology. In addition, there are increasing trials of drugs aimed at metabolism, inflammation, synapse, or neuronal protection, as well as vascular and epigenetic interventions (Cummings et al., 2020). Most trials have been aimed at preventing the formation of amyloid plaques (β-secretase inhibitors), counteracting aggregation, or increasing the breakdown of amyloid (anti amyloid immunotherapy) and preventing phosphorylation of tau (anti - tau immunotherapy).

Cramer et al in 2012 demonstrated that RXR agonists like bexarotene can enhance normal Aβ clearance mechanisms by activating PPARγ:RXR and LXR:RXR, inducing ApoE expression, facilitating Aβ clearance, and promoting microglial phagocytosis. Development of new drugs against Alzheimer's disease is the need of the hour and using existing drugs like bexarotene which have proven efficacy can reduce the clinical trial

time. Furthermore, evaluating the potential of novel analogs of bexarotene for treatment of AD could prove to be a useful tool to combat AD. Therefore, a second aim of this thesis is to describe the development and testing of a new generation of bexarotene analogs that may serve as potent inducers of ApoE, while limiting the side effects of this class of drugs, and to thus enhance Aβ clearance and serve as efficacious therapeutics in the treatment of Alzheimer's disease.

CHAPTER 2

1. GENERATION 6, 7, 8, 9, 10 AND 11 ANALOGS (INTRODUCTION, STRUCTURE, METHODOLOGY)

Between 2021 and 2023 our research group reported the synthesis and evaluation of Generation 6, 7, 8, 9, 10 and 11 rexinoids (A64-A126) as novel analogs of bexarotene, and several of these analogs exhibited distinctive activity profiles and promoted a range of gene expression in vitro. When our group first undertook research on rexinoids, we reported a fluorinated bexarotene analog (A18) (Wagner et al., 2009) followed by other halogenated, and even a difluorinated bexarotene analog (A28) (Furmick et al., 2012). The pyridine bexarotene analog (A33) (Boehm et al., 1995) and the pyrimidine bexarotene analog (A35) (Jurutka et al., 2013), as well as LGD100268 (A30) (Boehm et al., 1995) and the LGD100268 pyrimidine analog (A40) (Jurutka et al., 2013) all exhibited an increase in RXR activity compared to bexarotene and superior therapeutic effects in mouse models of cancer (Boehm et al., 1994 ; Liby et al., 2006). Introducing an unsaturation in bexarotene's aliphatic ring results in compound (A32) (Zhang et al., 1995; Faul et al., 2001), and the unsaturated-fluorinated bexarotene (A41) (Jurutka et al., 2013) also activates LXR (Marshall et al., 2015). CD3254 A34 (Santin et al., 2009) and CD2915 (A36) (Gianni et al., 2000) are two synthetic rexinoids with activity comparable to bexarotene. Our group utilized A34 and A36 to design analogous rexinoids A31, A39, A37 and A38 (Jurutka et al., 2013). Kakuta and colleagues reported compound A54 (NEt-TMN) (Fujii et al.,2010; Ohsawa et al., 2010; Kakuta et al., 2012;

Ohsawa 2013) as well as its analogous compounds A63 (Kagechika et al., 1998;, Ohta et al., 1998; ,Ohta et al., 2011) and A61 **(**Kagechika et al., 1998;, Ohta et al., 1998)—all of which showed high potency and selectivity for RXR alongside many other NEt-TMN derivatives that our group reported (Heck et al.,2016). Even replacing the ethyl group with a methyl group on the linking nitrogen atom of NEt-TMN leads to potent rexinoids such as A50 (Ohta et al., 2000), A48 (Ohta et al., 2000), and A64 (Ohta et al., 2000) (Figure 4).

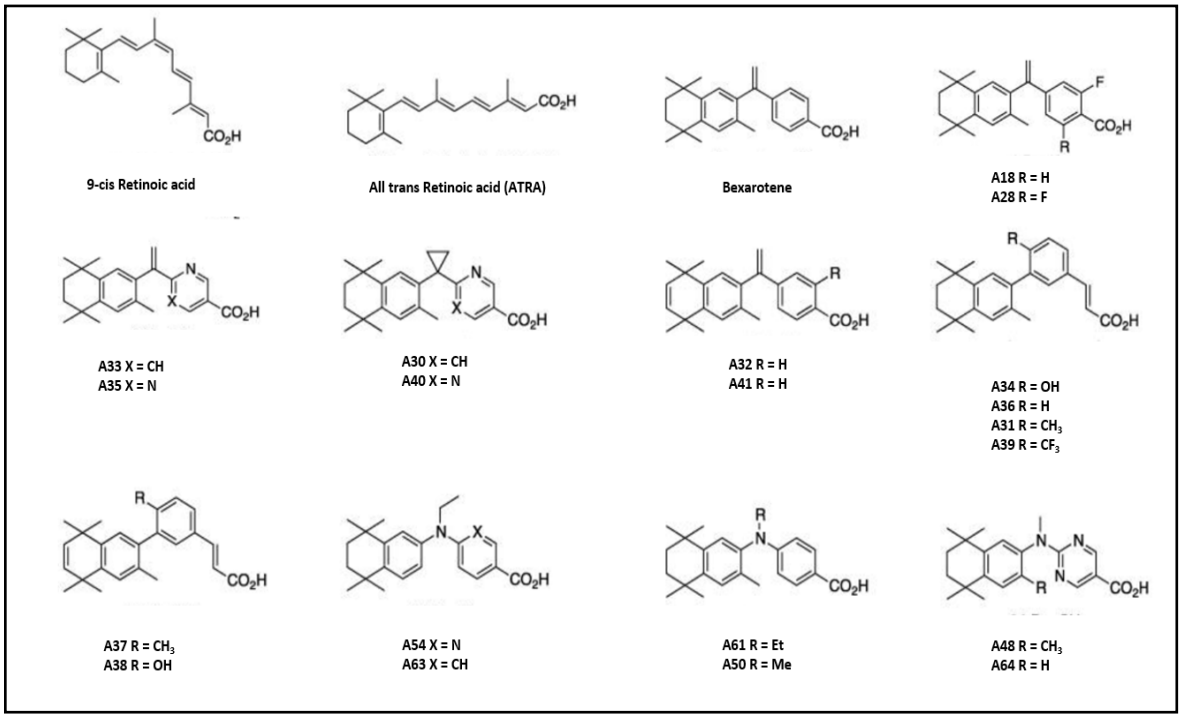

Figure 4: Structures Of 9-*cis* Retinoic Acid, ATRA, Bexarotene and Rexinoids

Generation 6 Rexinoids

Generation 6 rexinoids (Fig. 5) consists of four novel analogs of 6-(ethyl)(4-isobutoxy-3-isopropylphenyl)amino nicotinic acid or NEt-4IB, analogs A65-A69 and a partial analog of NEt-4IB, analog A64. A64 (Kawata et al., 2015) is described by Kakuta's group as a potent partial RXR agonist exhibiting promise in mouse models of diabetes and pulmonary emphysema. We were interested in testing the activity of A64 in vitro, since we were expecting a reduced side-effect profile via RXR-dependent cross-signaling for these types of compounds (Jurutka et al., 2021).

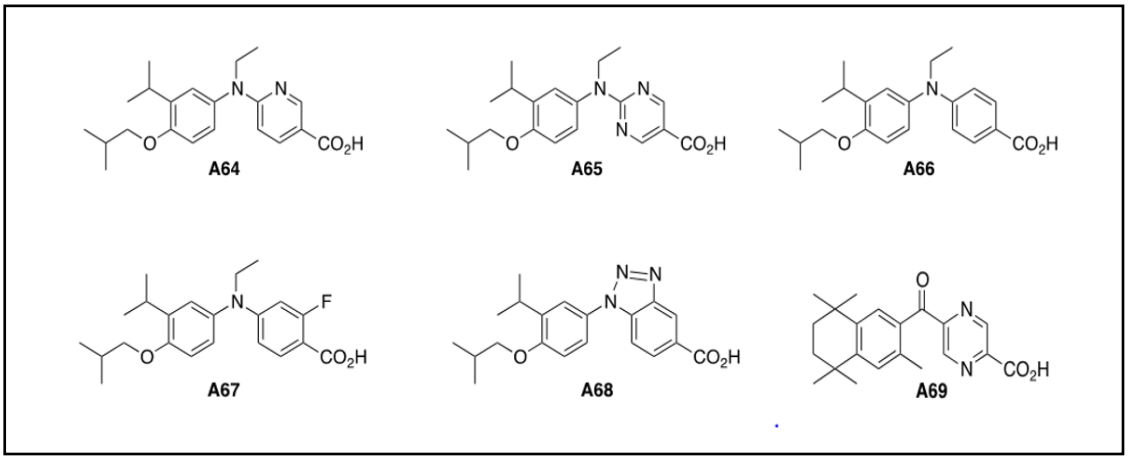

Figure 5: Structures Of Generation 6 Rexinoids

Generation 7 Rexinoids

Generation 7 rexinoids (Fig. 6) consist of seven novel analogs of 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahyro-2-naphthyl)ethynyl]benzoic Acid commonly referred to as bexarotene, and two analogs of analog 64**.** Analogs A70-A74, A77 and A78 are the novel

analogs of bexarotene and analogs A76 and A75 are the analogs of analog 64. We were interested in testing the activity of analogs A76 and A75 in vitro, since we were expecting a reduced side-effect profile via RXR-dependent cross-signaling for these types of compounds (Jurutka et al., 2021).

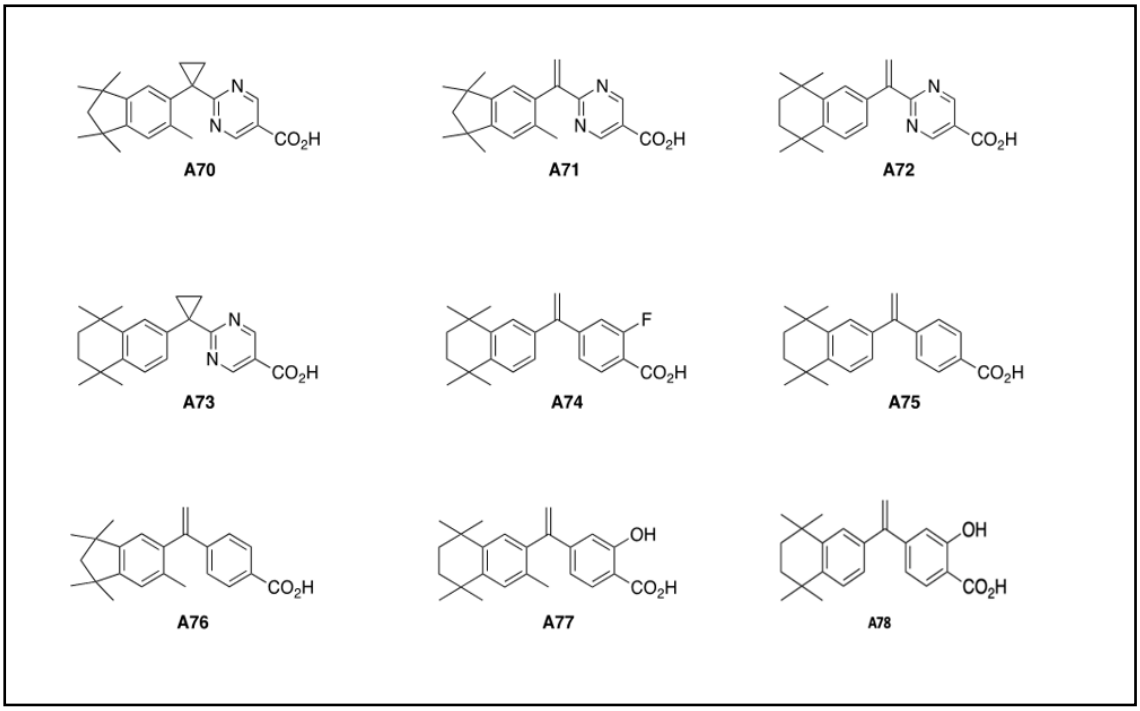

Figure 6: Structure Of Generation 7 Rexinoids

Generation 8 Rexinoids

Generation 8 rexinoids (Fig. 7), analogs A79-A83 were synthesized by using many of the compounds shown in Figure 4 as starting points to analyze how altering the structure of a compound by introducing new modifications result in changes to the compound's activities. For example, we were interested in substituting an isochroman group for the aliphatic ring system in bexarotene and some of the CD3254 analogs that have been

described, as well as a multiple fused aryl-ring system for synthesis of analogs A79-A83 (Jurutka et al., 2022).

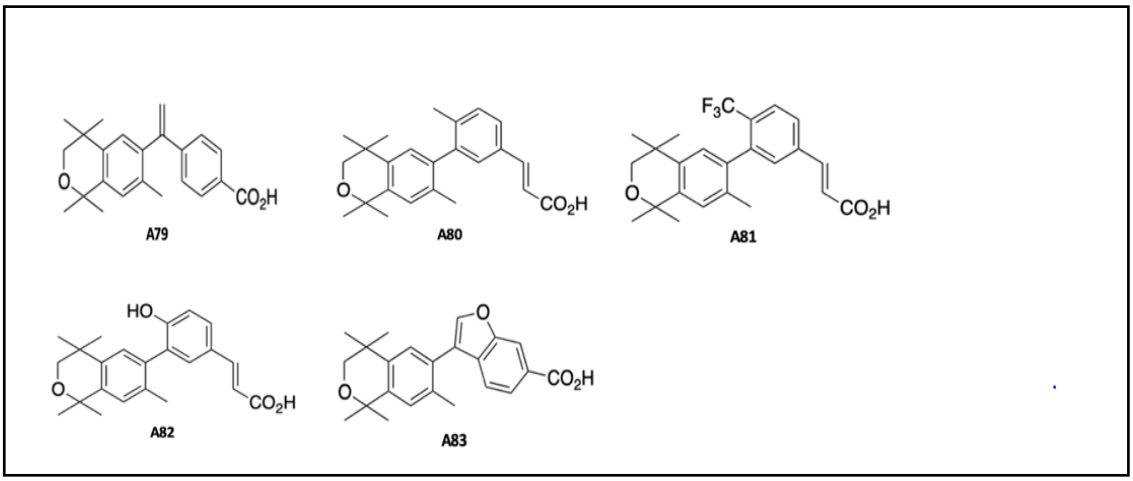

Figure 7: Structure Of Generation 8 Rexinoids

Generation 9 Rexinoids

Generation 9 rexinoids (Fig. 8) consist of analogs A84-A91**.** The analogs were synthesized by modifying the structure of compounds A50, A48, A64 and compound A54 and its various analogs. Analog A84 was synthesized by a pyrimidine aromatic ring substitution from compounds A50, A48 and A64. Analogs A89, A88, A91, A87 were synthesized by introducing different modifications such as substituting an allyl group, various aromatic rings, addition of a methyl group into the analogs of NEt-TMN (Jurutka et al., 2022). Analogs A90, A86 and A85 were formed because of substitution of ethyl group with a methyl group on the linking nitrogen atom of NEt-TMN (Ohta et al., 2000) (Fig. 4).

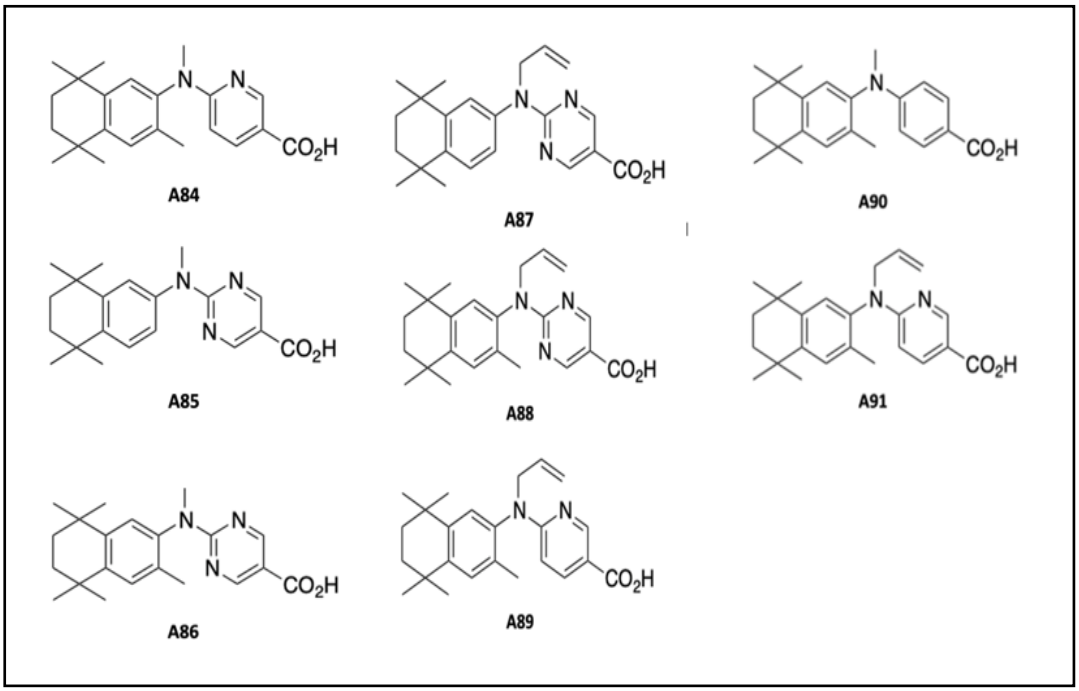

Figure 8: Structure Of Generation 9 Rexinoids

Generation 10 Rexinoids

Generation 10 rexinoids (Fig. 9) consist of analogs A92-A100. The analogs were synthesized by modifying the structure of NEt-TMN and substituting the isochroman group for the aliphatic ring system and then varying the *N*-alkyl chains—including methy, ethyl and allyl—along with different aromatic acid ring systems (Jurutka et al., 2022). Interestingly, compound **A72 (**Kagechika et al., 1998) was previously made and reported by Kagechika and co-workers, so we were interested in synthesizing many possible analogs of it (**compounds A94**, **A97**, and **A100**) and comparing their activities.

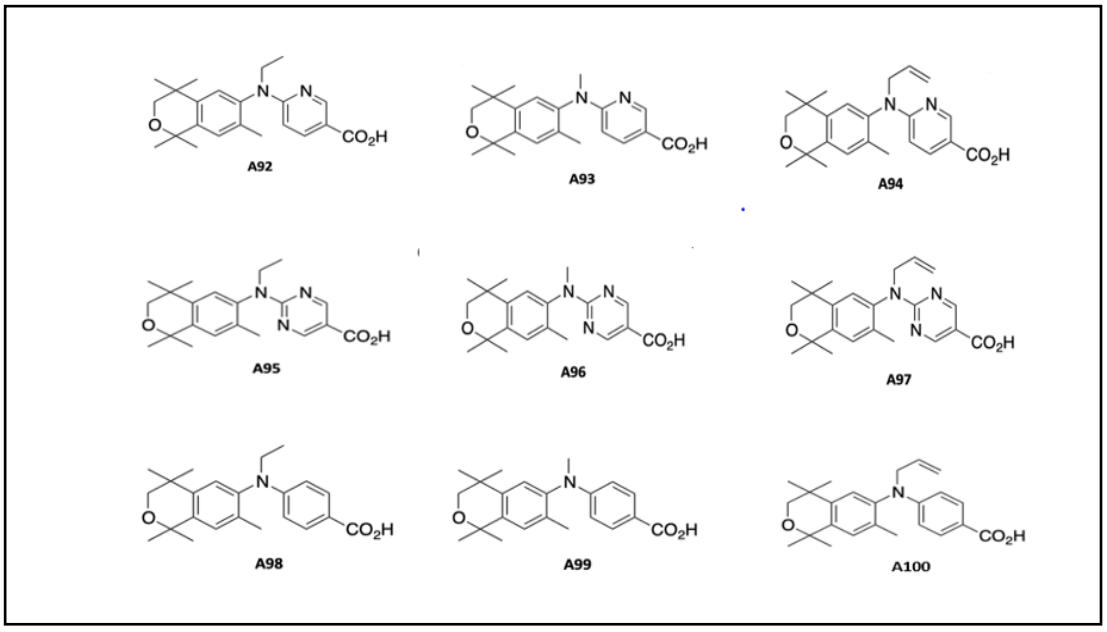

Figure 9: Structure Of Generation 10 Rexinoids

Generation 11 Rexinoids

Generation 11 rexinoids (Fig. 10) consist of analogs A101-A126. For the majority of the Generation 11 compounds (A101-A124), we basically wanted to assess how substituting a 1,1,4,4,6-pentamethyl-1,4-dihydronaphthalenyl system for the 1,1,4,4,6-pentamethyl-1,2,3,4-tetrahydronaphthalenyl system of NEt-TMN, in addition to varying the alkyl group and then the aromatic ring system that bears the carboxylic acid would affect the activity. For the last two compounds, we substituted the isochroman ring system for the 1,1,4,4,6-pentamethyl-1,2,3,4-tetrahydronaphthalenyl system of LDG100268 and a

known pyridine-bexarotene analog to generate A125 and 126, respectively.

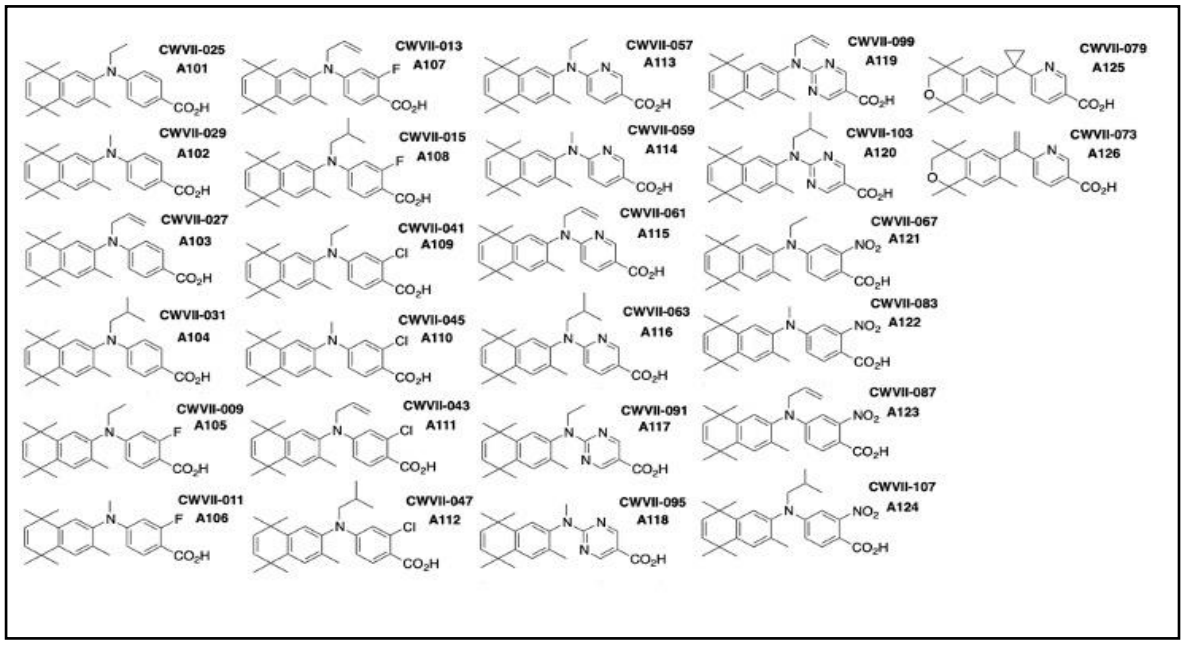

Figure 10: Structure Of Generation 11 Rexinoids

Materials and Methods

Mammalian-2-Hybrid assay

Human glioblastoma cells (U87) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and used to perform the Mammalian-2-Hybrid assays with Generation 8, 9 and 10 analogs and HEK-293 cells were used to perform the M2H assay with Generation 6, 7 and 11 analogs. Authentication and validation of these master stocks included both testing for mycoplasma contamination (via Universal Mycoplasma Detection Kit; ATCC, Cat. #30-1012K), as well as short tandem repeat (STR) analysis to confirm cell line identity. For M2H assays, U87 glial cells and HEK-293 cells were seeded at a density of 80,000 cells/well for Generation 6 and 7 analogs and at 60,000 cells/well for Generation 11 analogs in a 24-well plate. The cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 37 degrees Celsius, 5% CO2 for 24 h. The cells in each individual well were transiently transfected using 1.25µl Polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's protocol. The cells were cotransfected using 50ng pCMV-hRXR binding domain vector (BD) and 50ng pCMV-hRXR activation domain (AD), as well as with 250 ng PFR-Luc reporter gene containing BD-binding sites and 20ng renilla control plasmid. After 22–24 h of transfection, the cells were treated with either vehicle control ethanol, reference compound bexarotene (100 nM) or 100 nM of the indicated bexarotene analog. All compounds were solubilized in ethanol. After 24 h of treatment, the cells

were lysed in 1X passive lysis buffer (Promega, Madison, WI USA) and the amount of reporter gene product (luciferase) was quantified using the Dual-Luciferase Reporter Assay System based on the manufacturer's protocol (Promega) in a Sirius FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). Luminescence resulting from the inducible firefly luciferase was divided by luminescence from the constitutively expressed Renilla luciferase to normalize for transfection efficacy, cell death, and cellular toxicity from ligand exposure. The data are a compilation of between six to eight independent assays with each treatment group dosed in quadruplicate for each independent assay. The activity of the reporter gene was measured in comparison to the reference compound bexarotene set to 100%. Error bars on all graphs indicate the standard deviation of the replicate experiments.

RXRE assay

For RXRE assays, U87 glial cells (Generation 8, 9 and 10 analogs) and HEK-293 cells (Generation 6, 7 and 11 analogs) were seeded at a density of 80,000 cells/well for Generation 6 and 7 analogs and at 60,000 cells/well for Generation 11 analogs in a 24-well plate. The cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 37 degrees Celsius, 5% CO2 for 24 h. The cells in each individual well were transiently transfected using 1.25µl Polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's protocol. The cells were cotransfected with 25 ng RXRα, 250ng RXRE –luciferase reporter gene and 20ng renilla

control plasmid. After 22–24 h of transfection, the cells were treated with either vehicle control ethanol, reference compound bexarotene (100 nM) or 100 nM of the indicated bexarotene analog. All compounds were solubilized in ethanol. After 24 h of treatment, the cells were lysed in 1X passive lysis buffer (Promega, Madison, WI USA) and the amount of reporter gene product (luciferase) was quantified using the Dual-Luciferase Reporter Assay System based on the manufacturer's protocol (Promega) in a Sirius FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). Luminescence resulting from the inducible firefly luciferase was divided by luminescence from the constitutively expressed Renilla luciferase to normalize for transfection efficacy, cell death, and cellular toxicity from ligand exposure. The data are a compilation of between six to eight independent assays with each treatment group dosed in quadruplicate for each independent assay. The activity of the reporter gene was measured in comparison to the reference compound bexarotene set to 100%. Error bars on all graphs indicate the standard deviation of the replicate experiments.

LXRE assay

For LXRE experiments, U87 cells were seeded at a density of 80,000 cells/well in a 24-well plate and maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 37 degrees Celsius, 5% CO2 for 24 h. The cells were transiently transfected in individual wells using Polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's protocol. The cells in each well received 250 ng of

an LXRE-luciferase reporter gene, 50 ng of CMX-h-LXRα (an expression vector for human LXRα), 50 ng of pSG5-human RXRα (an expression vector for human RXRα), and 20 ng of Renilla control plasmid was used along with 1.25 µL of PEI reagent. After 22–24 h of transfection, the cells were treated with either vehicle control ethanol, reference compound bexarotene (100 nM) alone, or in combination with 100 nM T0901317 (an LXR ligand), or 100 nM of the indicated bexarotene analog either alone or in combination with T0901317, as indicated. All compounds were solubilized in ethanol. After 24 h of treatment, the cells were lysed in 1X passive lysis buffer (Promega, Madison, WI USA) and the amount of reporter gene product (luciferase) was quantified using the Dual-Luciferase Reporter Assay System based on the manufacturer's protocol (Promega) in a Sirius FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). Luminescence resulting from the inducible firefly luciferase was divided by luminescence from the constitutively expressed Renilla luciferase in order to normalize for transfection efficacy, cell death, and cellular toxicity from ligand exposure. The data are a compilation of between six to eight independent assays with each treatment group dosed in triplicate for each independent assay. The LXRE-directed transcriptional activation of the reporter gene was measured in comparison to the reference compound bexarotene set to 100%. Error bars on all graphs indicate the standard deviation of the replicate experiments.

RARE assay

Human embryonic kidney cells (HEK293) were plated at 60,000 cells per well in a 24-well plate and maintained as described above. After 22–24 h, the cells were transiently transfected with 250 ng pTK-DR5(X2)-Luc, 25 ng pSG5-human RXRα, and 20 ng of Renilla control plasmid using 1.25 µL polyethylenimine (PEI) per well for 24 h. The sequence of the double DR5 RARE is: 5'-AAAGGTCACCGAAAGGTCACCATCCCGGGAGGTCACCGAAAGGTCACC-3' (DR5 responsive elements underlined). After 22–24 h of transfection, the cells were treated with ethanol vehicle (0.1%), all-trans-retinoic acid (ATRA, the endogenous ligand for RAR), or the indicated rexinoid analog at a final concentration of 10 nM. After 24 h of treatment, the cells were lysed and the retinoid activity was measured as described above (dual luciferase assay). The activity of bexarotene (or analog) divided by the activity of ATRA (expressed as a percentage) represents the RARE activity. The data are a compilation of between three to four independent assays with each treatment group dosed in triplicate for each independent experiment. The value for the positive control ATRA was set to 100%.

Quantitative Real-Time PCR (Hut78 RNA)

Human cutaneous T-lymphocyte (Hut78) cells were maintained in DMEM/high glucose, L-glutamine, and sodium pyruvate (Cytiva Hyclone) containing 10% FBS (Atlanta Biologicals), supplemented with 100 µg/mL streptomycin and 100 units/mL penicillin

(Gibco) at 37 degrees Celsius, 5% CO 2. Cells were plated at 400,000 cells per well in a 6-well plate. After 24 h, the medium was replaced with DMEM containing 1% FBS by centrifugation of cells at 200g for 10 minutes. The cells were dosed in DMEM 1% FBS containing ethanol, DMSO, bexarotene, or analog at a final concentration of 100 nM for 24 h. Total RNA was isolated from each well using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The RNA obtained was quantified using A260/280 spectrophotometry. DNase-treated total RNA (0.1 μg) was reverse-transcribed via the use of the AzuraQuant Green 1-Step qPCR Mix LoRox 1,000 Reactions kit (*Azura Genomics*) to prepare 62 μL of first-strand cDNA synthesis and real-time PCR components. Reactions were prepared by adding 31 μL of 2x AzuraQuant 1-step Green LoRox, 1.55 μL of forward/reverse primers (18 μM), 3.1 μL of 20x AzuraSprint Rtase, DNase-treated total RNA (0.1 μg), and PCR-grade water for a total volume of 10 μL per well. Reactions were performed in 96-well plates in a BioRad CFX96 thermal cycler using a 40-cycle profile. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as $2^{-\Delta\Delta Ct}$ according to Applied Biosystems' User Bulletin 2, revision B, "Relative Quantitation of Gene Expression". The primers utilized during PCR experimentation are as follows:

human GAPDH forward, 5′ACAACTTTGGTATCGTGAAGGAC3′

human GAPDH reverse, 5′-CAGGGATGATGTTCTGGAGAGC-3′

human EGR3 forward 5’-CAATCTGTACCCCGAGGAGA-3’

human EGR3 reverse 5’-GGAAGGAGCCGGAGTAAGAG-3’

human ATF3 forward 5’-GAGGATTTTGCTAACCTGACGC-3’

human ATF3 reverse 5’-CTACCTCGGCTTTTGTGATGG-3’.

Quantitative Real-Time PCR (U87 glial cells)

Human glioblastoma (U87) cells were maintained in DMEM as described above. The cells were plated at 300,000 cells per well in a six-well plate and immediately dosed with ethanol, bexarotene + TO, or analog +TO at a final concentration of 100 nM for 24 h. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) as per manufacturer's protocol. DNase-treated total RNA (0.1 μg) was reverse-transcribed via the use of the AzuraQuant Green 1-Step qPCR Mix LoRox 1,000 Reactions kit (*Azura Genomics*) to prepare 62 μL of first-strand cDNA synthesis and real-time PCR components. Reactions were prepared by adding 21 μL of 2x AzuraQuant 1-step Green LoRox, 1.05 μL of forward/reverse primers (18 μM), 2.1 μL of 20x AzuraSprint Rtase, DNase-treated total RNA (0.1 μg), and PCR-grade water for a total volume of 10 μL per well. Reactions were performed in 96-well plates in a BioRad CFX96 thermal cycler using a 40-cycle profile. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as $2^{-\Delta\Delta Ct}$ according to Applied Biosystems’ User Bulletin 2, revision B,

"Relative Quantitation of Gene Expression". The primers utilized during PCR experimentation are as follows:

human GAPDH forward, 5′ACAACTTTGGTATCGTGAAGGAC3′

human GAPDH reverse, 5′-CAGGGATGATGTTCTGGAGAGC-3′

human apolipoprotein E (ApoE): forward, 5′-GGGTCGCTTTTGGGATTAC-3′

human apolipoprotein E (ApoE): reverse, 3′- CAACTCCTTCATGGTCTCG-5′

Data analysis.

Statistical analysis was performed using Microsoft Excel software. T-tests were performed, as appropriate. All error bars represent the standard deviation. Data points without error bars have standard deviations below Excel's limit to display. Statistical differences between two groups (generally the bexarotene control group versus bexarotene analog group) were determined by a two-sided Student's t-test. A p-value of less than or equal to 0.05 was considered significant.

CHAPTER 3

Abstract:

Five novel analogs of 6-(ethyl)(4-isobutoxy-3-isopropylphenyl)amino)nicotinic acid—or NEt-4IB—in addition to seven novel analogs of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene) were prepared and evaluated for selective retinoid-X-receptor (RXR) agonism alongside bexarotene (1), a FDA-approved drug for cutaneous T-cell lymphoma (CTCL). Bexarotene treatment elicits side-effects by provoking or disrupting other RXR-dependent pathways. Analogs were assessed by the modeling of binding to RXR and then evaluated in a human cell-based RXR-RXR mammalian-2-hybrid (M2H) system as well as a RXRE-controlled transcriptional system. The analogs were also tested in KMT2A-MLLT3 leukemia cells and the EC50 and IC50 values were determined for these compounds (Data not included in the thesis). Moreover, the analogs were assessed for activation of LXR in an LXRE system as drivers of ApoE expression and subsequent use as potential therapeutics in neurodegenerative disorders, and the results revealed that these compounds exerted a range of differential LXR-RXR activation and selectivity. Furthermore, several of the novel analogs in this study exhibited reduced RARE cross-signaling, implying RXR selectivity. These results demonstrate that modification of partial agonists such as NEt-4IB and potent rexinoids such as bexarotene can lead to compounds with improved RXR selectivity, decreased cross-signaling of other RXR-dependent nuclear receptors, increased LXRE-heterodimer

selectivity, and enhanced anti-proliferative potential in leukemia cell lines compared to therapeutics such as 1.

GENERATION 6 AND 7 ANALOGS (RESULTS AND DISCUSSION)

Biological Evaluation of Generation 6 Analogs (A64-A69) via an M2H Luciferase-based System.

In order for a rexinoid to carry out its therapeutic effects as a modulator of genes involved in cell differentiation, proliferation, and apoptosis, it must first induce RXR-RXR homodimerization (Panchal and Scarisbrick., 2015). Therefore, a mammalian-2-hybrid (M2H) luciferase assay was employed to determine the efficacy of RXR-RXR homodimerization induced by our analogs compared to bexarotene. In this assay, human embryonic kidney cells were transfected with the plasmid components of the M2H system (see Methods, Chapter 2), and the cells were subsequently dosed with either ethanol (vehicle), 100 nM bexarotene, or the indicated analog. After 24 hours of ligand exposure, the transcription of the luciferase gene, which is directly proportional to the degree of RXR-RXR homodimerization, was measured via a luminescence assay. Due to structural/chemical differences in our novel generation of analogs, the compounds were separated into "functional" Generation 6 (A64-A69) and Generation 7 (A70-A77) groupings. In this set of experiments, homodimerization and subsequent transcriptional activity of generation 6 analogs was compared to bexarotene, which was set to 100%. Transcriptional activity of our analogs ranged from 7.5% to 14.1% of the bexarotene control, with A64 displaying the greatest agonist activity in this set (Figure 11A).

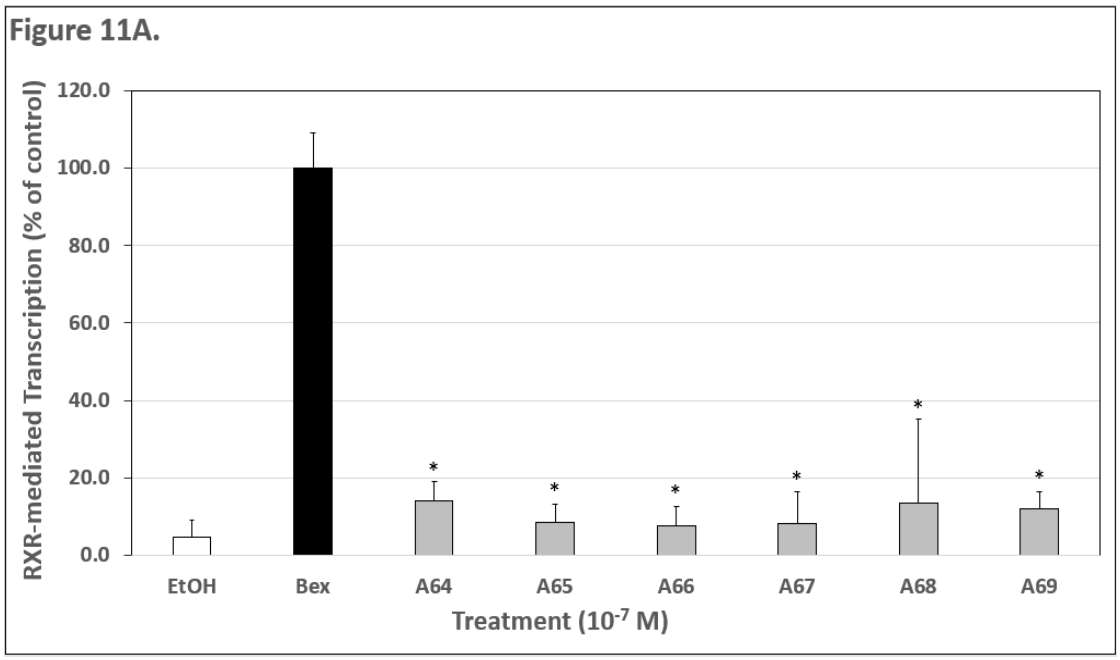

Figure 11. A: Biological Evaluation Of Generation 6 (A64-A69) RXR Agonists Via An M2H Luciferase-Based System. Human embryonic kidney cells (HEK293) were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%). Values are means ± SD with all analogs tested displaying lowered RXR homodimerization activity vs. bexarotene ($p < 0.05$).

Biological Evaluation of Generation 7 Analogs (A70-A77) via an M2H Luciferase-based System.

In a parallel set of experiments, we employed the same M2H luciferase assay as described above, but instead compared our Generation 7 analogs to bexarotene, which was again set to 100%. Transcriptional activity of our analogs ranged from 53.3% to 299.4% of the bexarotene control. Analogs A71, A72, and A75-A77 all outperformed

bexarotene (Figure 11B). Of these, A75-A77 displayed statistically significant ($p < 0.01$) improvement in driving RXR-RXR homodimerization compared to bexarotene with analog A77 exhibiting the highest activity of all compounds tested.

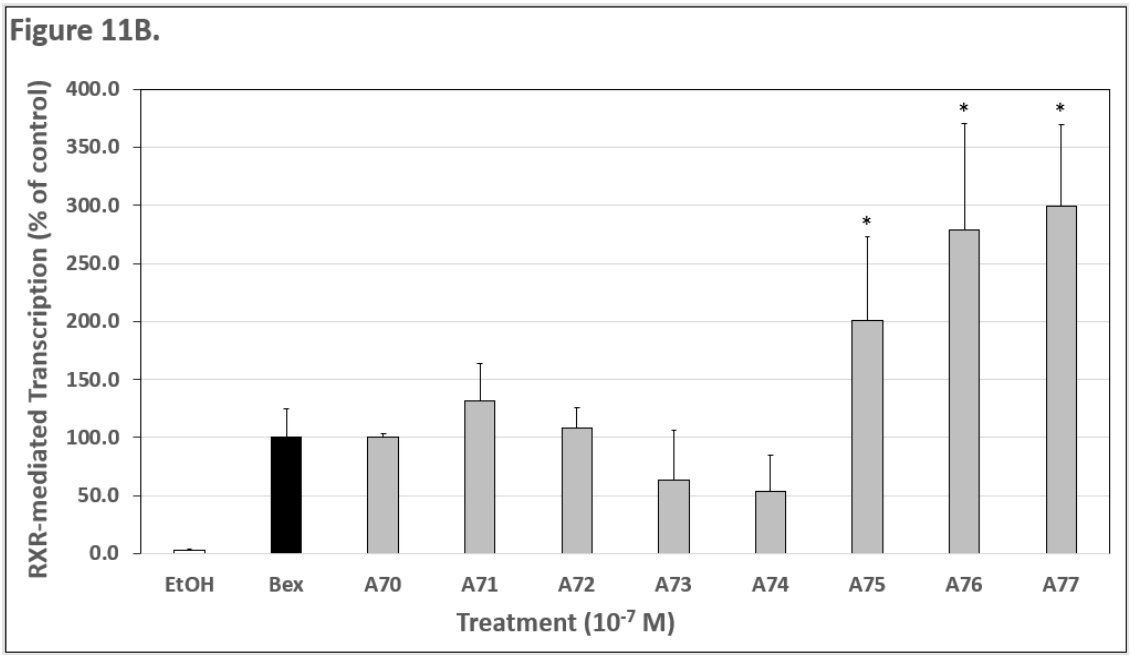

Figure 11. B: Biological Evaluation Of Generation 7 (A70-A77) RXR Agonists Via An M2H Luciferase-Based System. Human embryonic kidney cells (HEK293) were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%). Values are means ± SD with A75-A77 displaying enhanced RXR homodimerization activity vs. bexarotene (*$p < 0.05$), whereas A71-A74 displayed comparable activity vs. bexarotene.

Assessment of Generation 6 Analogs via an RXRE Luciferase-based System.

After RXR-RXR homodimerization takes place, the complex must then associate with its respective response element, the RXRE, in order to carry out transcriptional regulation.

To assess this next molecular step in the transduction pathway of rexinoid signaling, we utilized an RXRE luciferase assay where transcription of the luciferase gene is directly proportional to RXR-RXR homodimer binding to RXRE. We again employed human embryonic kidney cells; this time transfected with a plasmid containing an authentic RXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 6 analogs was compared to bexarotene set to 100%. In this set of experiments, transcriptional activity of our analogs ranged from 9% to 28% of the bexarotene control (Figure 12A), with A64 possessing the highest activity in this grouping, similar to the results observed in the M2H assay (compare Figure 11A). Interestingly, the overall trend seen in the results of the M2H assay very closely mimics what was measured in the RXRE assay. This is an expected and reasonable observation, as RXR-RXR homodimerization and homodimer association to the RXRE are consecutive molecular functions necessary for rexinoids to produce their pharmacologic effects.

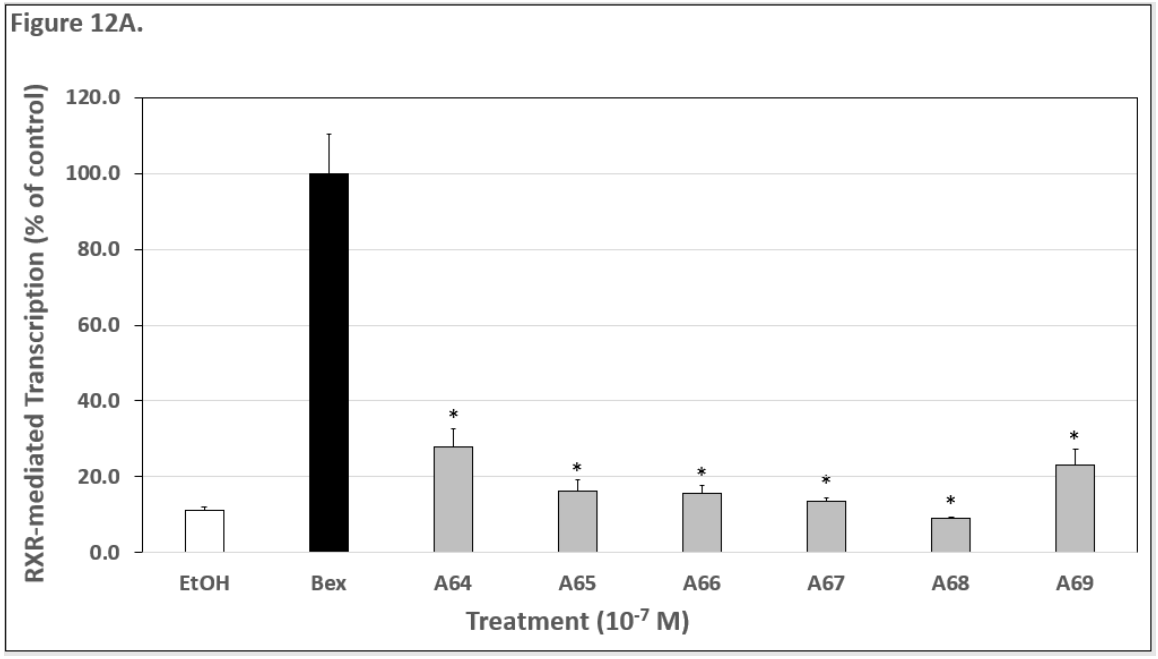

Figure 12. A : Biological Evaluation Of Generation 6 (A64-A69) RXR Agonists Via An RXRE Luciferase-Based System. Human embryonic kidney cells (HEK293) were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%). Values are means ± SD with A64-A69 displayed lowered activity vs. bexarotene (*$p < 0.05$).

Assessment of Generation 7 Analogs via an RXRE Luciferase-based System.

In this parallel set of RXRE experiments, we employed the RXRE assay and compared our Generation 7 analogs to bexarotene. Transcriptional activity of these analogs ranged from 94.7% to 246.3% of the bexarotene control (Figure 12B). Analogs A70, A75-A77 all displayed a greater activity trend than bexarotene, and A70, A76 and A77 revealed a statistically significant ($p < 0.05$) difference compared to the bexarotene control. Again,

the trend in these results closely mimics what was observed in the M2H assay, thus further validating both assay systems.

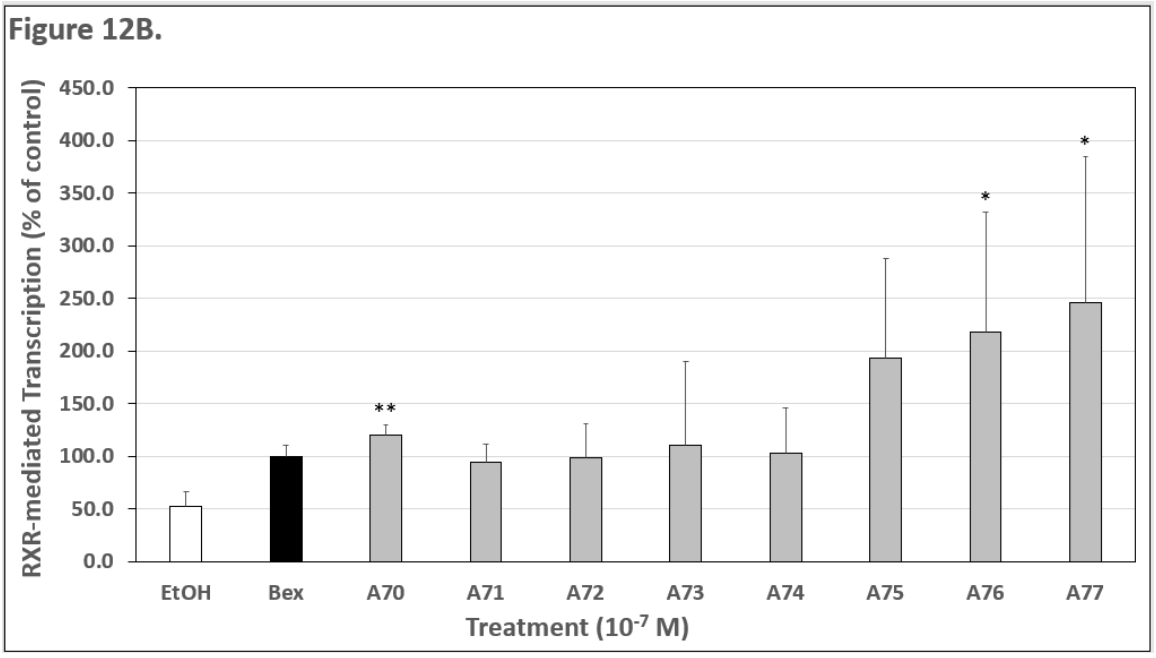

Figure 12. B: Biological Evaluation Of Generation 7 (A70-A77) RXR Agonists Via An RXRE Luciferase-Based System. Human embryonic kidney cells (HEK293) were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%). Values are means ± SD with A70, A76 and A77 displaying enhanced RXR-mediated transcriptional activity vs. bexarotene (*$p < 0.05$), whereas A72-A75 displayed comparable activity vs bexarotene.

Assessment of Generation 6 Analogs via an LXRE Luciferase-based System.

We next tested the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). We

utilized human embryonic kidney cells; this time transfected with a plasmid containing an authentic LXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 6 analogs was compared to bexarotene set to 100%. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or synergistic effects of dual ligand activation of the RXR-LXR heterodimer. In this set of experiments, transcriptional activity of our analogs ranged from 64.5% to 72.4% of the bexarotene control (Figure 13A). In this cohort, none of the analogs were found to more potent than bexarotene in activating LXRE.

Figure 13 A.

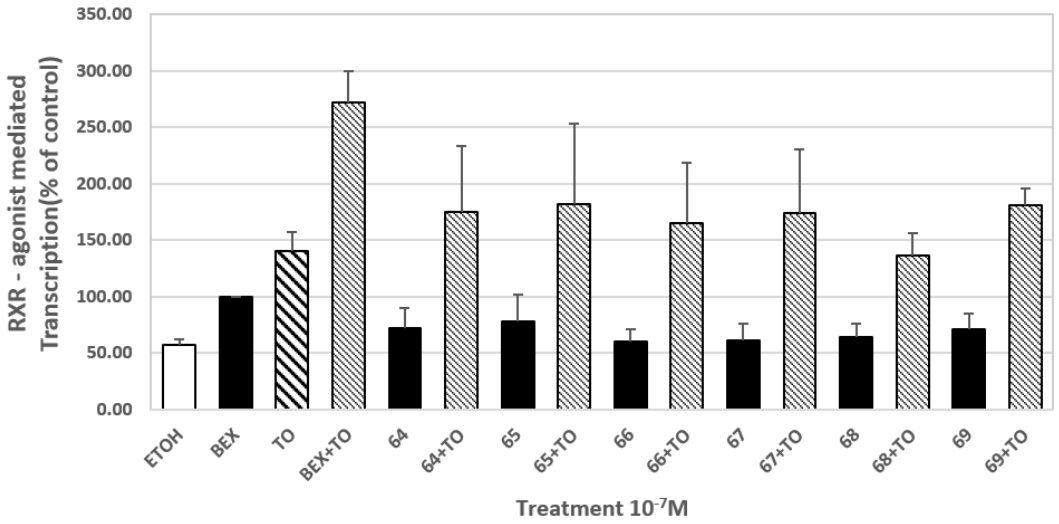

Figure 13. A: Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. HEK-293 human embryonic kidney cells were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to bexarotene, set to 100%.

While most of the analogs possessed slightly lower LXR activation when compared to bexarotene, it is important to consider this activity in the context of the RXR-RXR homodimer activity of each analog, and to thus "normalize" the LXR/LXRE heterodimer activation in order to yield a LXRE Heterodimer Specificity (LHS) score (Figure 13B). The results of this LHS analysis revealed that many of our novel compounds in Generation 6 (e.g., A64, A65, A66, A67, A68, A69) possessed greater LXRE/RXRE activity via increased heterodimer specificity than the parent bexarotene.

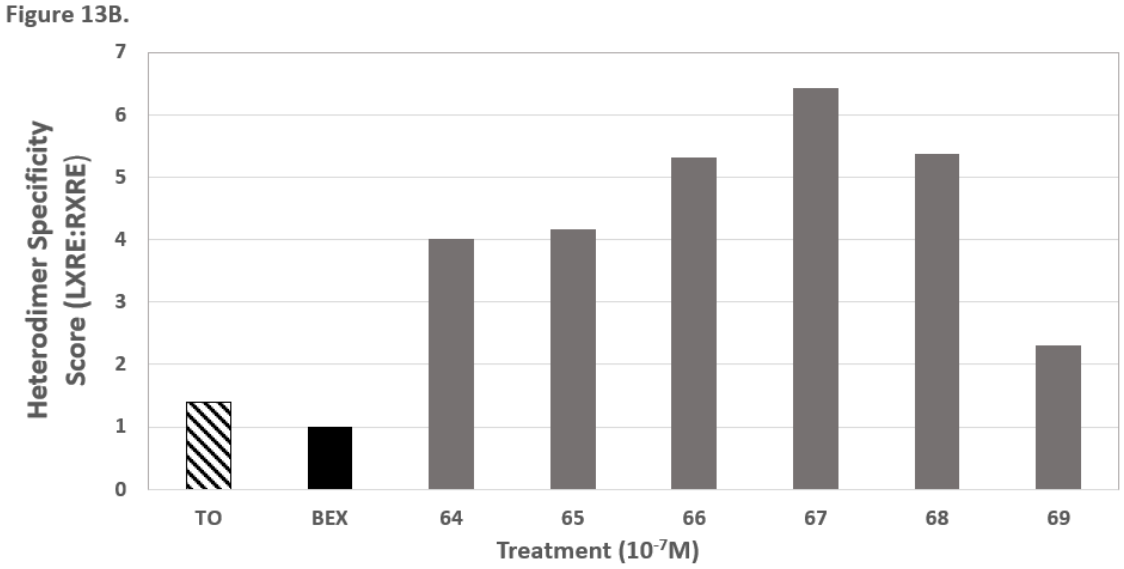

Figure 13. B: Heterodimer Specificity Score-The "Heterodimer Specificity Score" was determined by the LXRE:RXRE ratio with compound 1 set to 1.0. See text for definition.

Assessment of Generation 7 Analogs via an LXRE Luciferase-based System.

In this parallel set of LXRE experiments, we employed the LXRE assay and compared our Generation 7 analogs to bexarotene. Transcriptional activity of these analogs ranged from 46% to 91.1% of the bexarotene control (Figure 14A). The results of the LHS analysis revealed that two of our novel compounds in Generation 7 (A73 and A74) possessed greater LXRE/RXRE activity via increased heterodimer specificity than the parent bexarotene (Figure 14B).

Figure 14A.

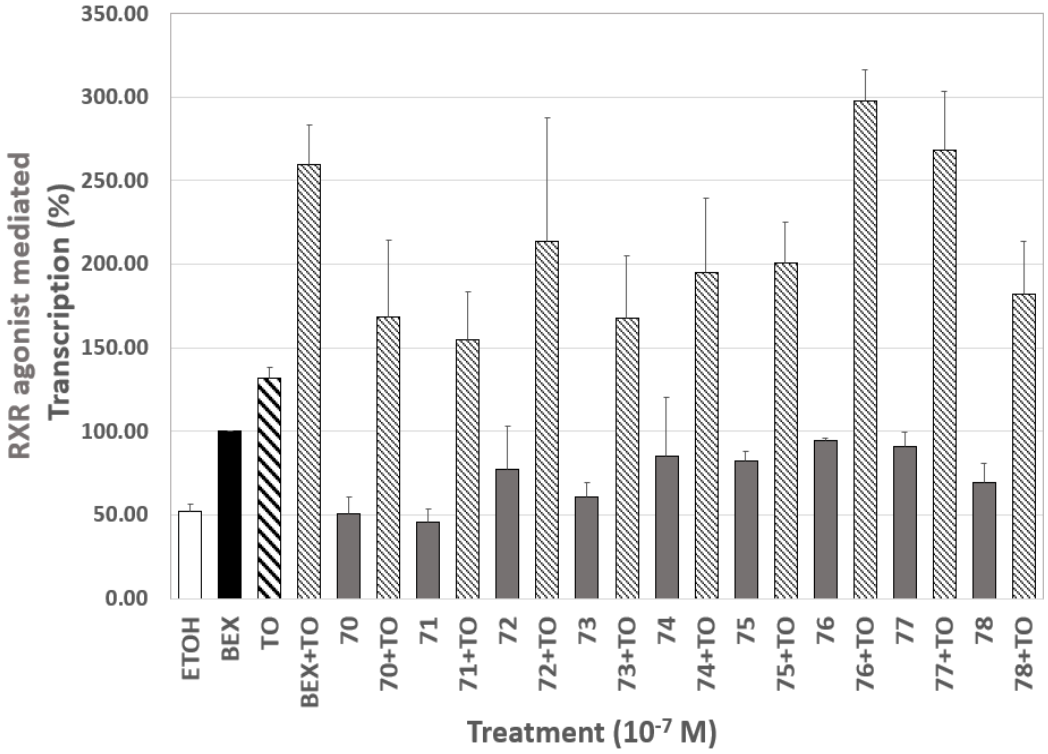

Figure 14. A : Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. HEK-293 human embryonic kidney cells were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to bexarotene, set to 100%.

Figure 14B.

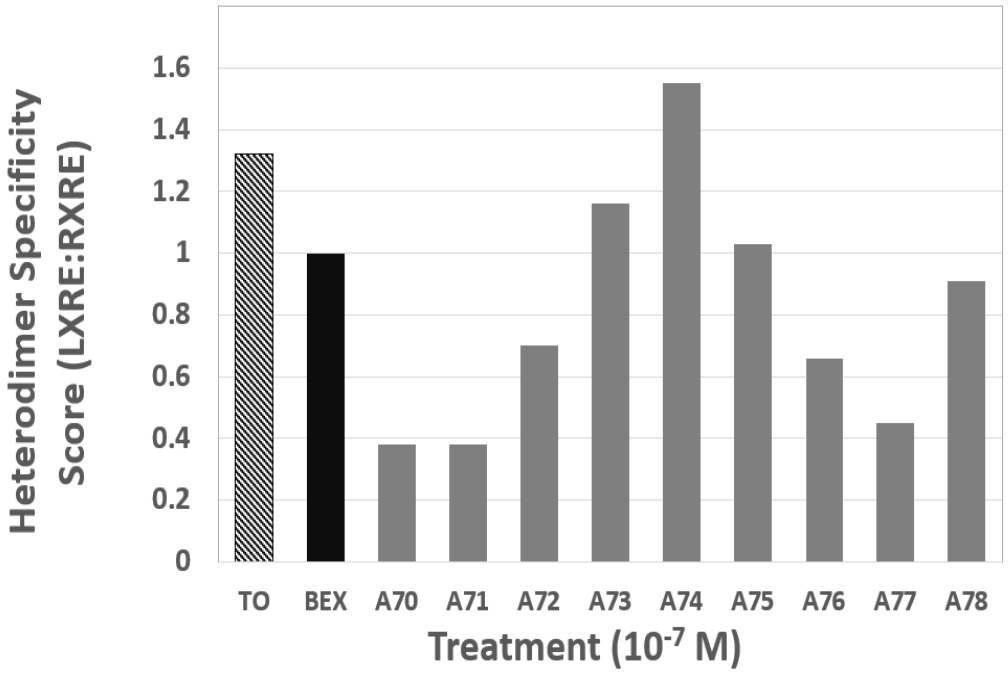

Figure 14. B: Heterodimer Specificity Score - The "Heterodimer Specificity Score" was determined by the LXRE:RXRE ratio with compound 1 set to 1.0. See text for definition.

Evaluation of Residual RARE activity of Generation 6 and 7 Analogs via RARE Luciferase-based System.

Since bexarotene is known to possess "residual" RARE activity, we evaluated the ability of Generation 6 compounds to induce transcription via the retinoic acid response element and retinoic acid receptor (RAR). Human embryonic cells (HEK293) were transfected with human RARα and dosed with 10 nM of either all-trans retinoic acid (RA), the natural ligand for RARα, bexarotene, or analogs. We found that bexarotene possessed and average 28.1% of the activity of the RA control (Figure 15). A66 showed the lowest RARE activity at 1.1%, which is indistinguishable from the ethanol control while as

A71 displayed the greatest RARE activation at 12.9% of RA. Thus, all of our novel analogs displayed significantly less "cross-over" onto RAR-RARE signaling compared to bexarotene.

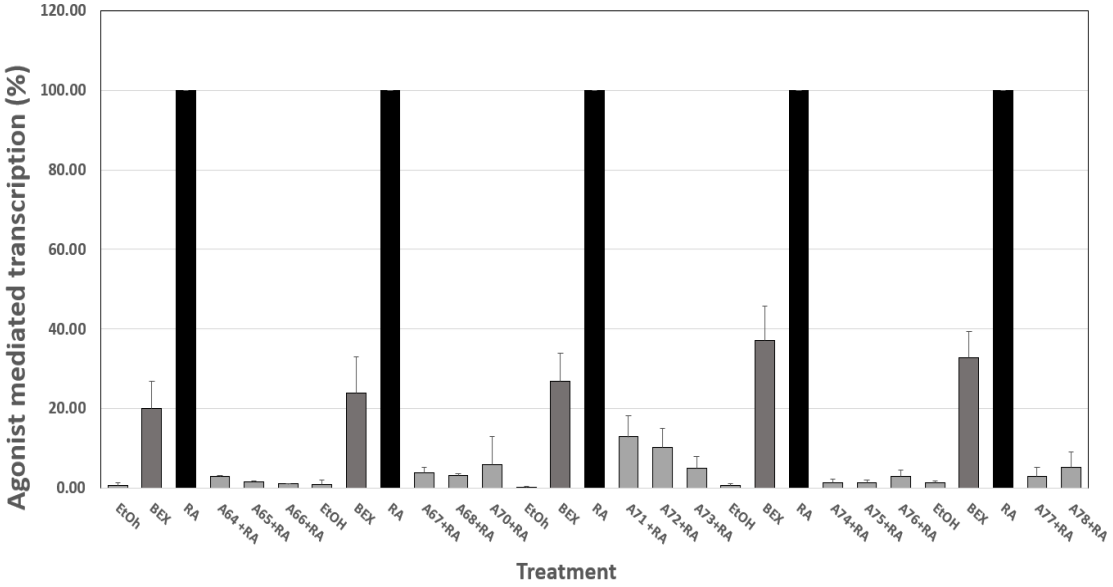

Figure 15: Assessment Of RXR Agonists Via An RARE-Luciferase Reporter Based Assay In Human Cells. Human embryonic cells (HEK293) were co-transfected with expression vectors for hRXRα, a RARE-luciferase reporter gene, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with bexarotene, analog, or all-trans-retinoic acid (RA) at 10 nM for 24 h. The RARE activity for RA was set to 100%. Values are means ± SD with all analogs tested displaying lowered RARE activity vs. compound 1 ($p < 0.05$).

qPCR Analysis of ATF3 Gene Induction.

qPCR analysis was performed to determine the efficacy of our analogs to upregulate gene transcription of the tumor suppressor gene, ATF3, relative to bexarotene. Since not all analogs displayed robust activity in the previous biological assays, only the most potent analogs, namely A75-A77, were selected for qPCR evaluation (Figure 16A). In this set of

experiments, bexarotene exhibited a mean 1.9-fold increase in ATF3 gene transcription relative to ethanol control. A75 and A76 yielded very similar fold inductions to bexarotene at 2.2 and 2.0, respectively. However, these differences were not statistically significant. A77, on the other hand, induced a mean fold increase of 12.8 over that of the ethanol control. Therefore, based on these results, which were performed in at least 3 independent biological replicates, A77 is 6.9 times more effective than bexarotene in the induction of ATF3, a statistically significant result (Figure 16A, $p < 0.001$).

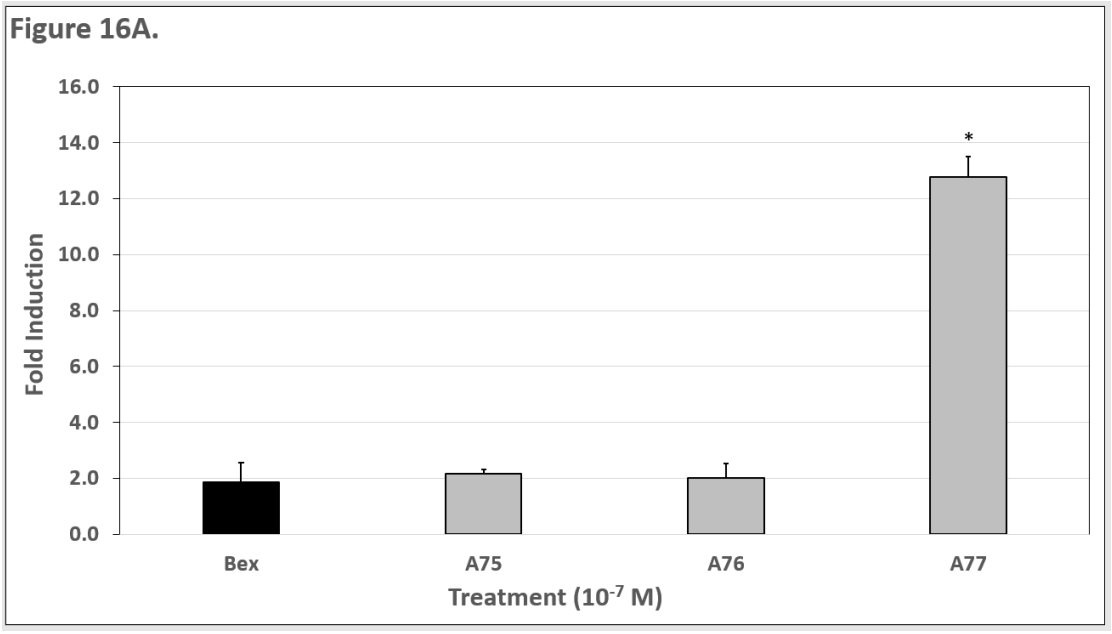

Figure 16. A: qPCR Analysis Of Our Most Potent RXR Agonists (A75-A77) To Induce ATF3 Gene Expression. Human cutaneous T-cell lymphoma (Hut78) cells were treated for 48 h with bexarotene or the indicated analog at 100 nM. A 0.1 μg portion of DNase-treated RNA was used for cDNA synthesis and subsequent qPCR. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as $2^{-\Delta\Delta Ct}$. * indicates $p < 0.001$ vs.

bexarotene. Results are from at least 3 independent biological replicates with multiple replicates in each experiment.

qPCR Analysis of EGR3 Gene Induction.

qPCR analysis was performed to determine the efficacy of our analogs to upregulate gene transcription of another known tumor suppressor gene, EGR3, relative to bexarotene. Again, only the analogs with the highest activity, namely A75-A77, were selected for qPCR evaluation (Figure 16B). In this set of experiments, bexarotene showed a mean 2.0-fold increase in EGR3 gene transcription relative to ethanol control. Analog A75 displayed a higher mean fold induction than bexarotene at 2.6, however this result did not reach full statistical significance. Additionally, A76 produced a lower mean fold induction than bexarotene at 1.5, but this result was also not statistically significant. In contrast, A77 induced EGR3 expression by 3.3-fold compared to the ethanol control which was also statistically significantly higher than bexarotene ($p < 0.05$). Therefore, A77 is 1.7 times more effective than bexarotene in the induction of EGR3.

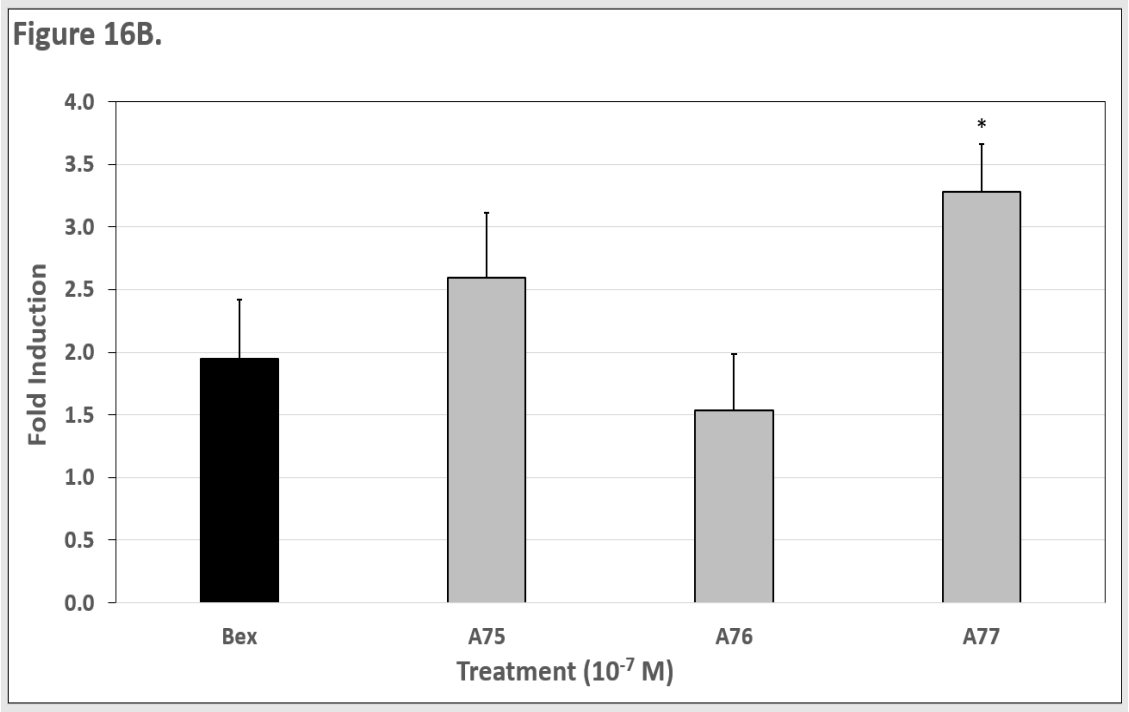

Figure 16. B: qPCR Analysis Of Our Most Potent RXR Agonists (A75-A77) To Induce EGR3 Gene Expression. Human cutaneous T-cell lymphoma (Hut78) cells were treated for 48 h with bexarotene or the indicated analog at 100 nM. A 0.1 μg portion of DNase-treated RNA was used for cDNA synthesis and subsequent qPCR. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as $2^{-\Delta\Delta Ct}$. * indicates $p < 0.05$ vs. bexarotene. Results are from at least 3 independent biological replicates with multiple replicates in each experiment.

Discussion

In this study, we describe the evaluation of fourteen novel analogs of bexarotene using various biological assays to assess RXR specificity and activity. The most potent compounds were also analyzed via qPCR to determine efficacy in the induction of two tumor suppressor genes, ATF3 and EGR3. Our analogs were synthesized as two distinct generations, Generation 6 and Generation 7, maintaining specific molecular motifs within their chemical structures. Based on the results obtained in our experiments, most of the Generation 6 analogs demonstrated less selectivity and potency as compared to bexarotene. In the M2H (Figure 11A) and RXRE (Figure 12A) assays, analogs A64-A69 yielded considerably less RXR-mediated transcription of the luciferase gene than bexarotene, indicating that these analogs are less effective at inducing a response through the RXR-RXRE nuclear signaling pathway. Rexinoids produce their chemotherapeutic effects, at least in part, via this pathway which permits the modulation of genes involved in cell proliferation, differentiation, and apoptosis. Therefore, analogs A64-A69 are less likely to lead to an improved therapeutic outcome in the treatment of CTCL than bexarotene. Conversely, Generation 7 analogs performed far more favorably. In this group, all analogs yielded either comparable or improved results compared to bexarotene across the M2H, RXRE, RARE, LXRE (Figures 11-16) and proliferation assays (data not shown). Most notably, analogs A75-A77 demonstrated an enhanced ability to induce RXR homodimerization (Figure 11B), activate RXRE (Figure 12B), and a reduced level of RAR "crossover". Therefore, we posit that the potential therapeutic utility of a rexinoid may be

predicted based on its performance in these assays and this approach may serve as a pragmatic tool in the assessment of novel rexinoids intended for the treatment of CTCL. With consideration given to this axiom, we further evaluated our top performing analogs, A75-A77, via qPCR to assess for upregulation of the ATF3 and EGR3 genes. These genes were specifically selected for analysis as they are involved in tumor suppression pathways and have been previously found to be upregulated in response to retinoids and rexinoids, such as bexarotene (Corona et al.,2016; Wang et al., 2019; Zhang et al., 2015). While upregulation of these genes may not be the only mechanism by which these compounds exert their chemotherapeutic effects, we hypothesized that our most potent analogs would generate similar or enhanced fold-inductions when compared to bexarotene. In fact, A75 and A76 produced equipollent responses while A77 produced statistically greater fold-inductions in both genes, especially ATF3 (Figure 16A and Figure 16B). These findings suggest that although bexarotene may be effective for the treatment of refractory CTCL, introducing new structural motifs to the bexarotene parent compound may in fact yield analogs that are more efficacious and maintain an improved side effect profile due to enhanced selectivity.

We also evaluated the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). LXR has been demonstrated to regulate lipid metabolism and inflammatory responses in the central nervous system, and there is ample evidence that robust cholesterol and lipid

metabolism in the brain (including enhanced ApoE expression) are critical to mitigating dementia. Biological evaluation of our novel RXR agonists for their ability to transactivate via an LXRE sequence that is found naturally in the promoter of LXR-RXR controlled genes, including ApoE, was carried out in human embryonic cells (HEK293) with bexarotene as a comparison. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or synergistic effects of dual ligand activation of the RXR-LXR heterodimer. The results (Figure 13A and Figure 14A) revealed that in comparison to the parent compound bexarotene alone, single dosing of the cells with any of the tested analogs displayed less LXR/LXRE activity although, some of the analogs were found to have increased heterodimer specificity as compared to Bexarotene indicating that the Generation 6 and 7 analogs have potential to activate LXRE mediated transcription. Thus, these analogs not only possess the potential for treating CTCL, as described above, but these compounds may also have some utility in the treatment of Alzheimer's disease given their ability to significantly and specifically activate the LXR-RXR heterodimer to potentially drive ApoE expression.

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CHAPTER 4

Abstract:

Bexarotene is an FDA-approved drug for the treatment of cutaneous T-cell lymphoma (CTCL); however, its use provokes or disrupts other retinoid-X-receptor (RXR)-dependent nuclear receptor pathways and thereby incites side effects including hypothyroidism and raised triglycerides. Two novel bexarotene analogs, as well as three unique CD3254 analogs and thirteen novel NEt-TMN analogs, were synthesized and characterized for their ability to induce RXR agonism in comparison to bexarotene (**1**). Several analogs in all three groups possessed an isochroman ring substitution for the bexarotene aliphatic group. Analogs were modeled for RXR binding affinity, and EC_{50} as well as IC_{50} values were established for all analogs in a KMT2A-MLLT3 leukemia cell line*. All analogs were assessed for liver-X-receptor (LXR) activity in an LXRE system to gauge the potential for the compounds to provoke raised triglycerides by increasing LXR activity, as well as to drive LXRE-mediated transcription of brain ApoE expression as a marker for potential therapeutic use in neurodegenerative disorders. Preliminary results suggest these compounds display a broad spectrum of off-target activities. However, many of the novel compounds were observed to be more potent than **1**. While some RXR agonists cross-signal the retinoic acid receptor (RAR), many of the rexinoids in this work displayed reduced RAR activity. The isochroman group did not appear to substantially reduce RXR activity on its own. The results of this study reveal that modifying potent, selective rexinoids like bexarotene, CD3254, and NEt-TMN can

provide rexinoids with increased RXR selectivity, decreased potential for cross-signaling, and improved anti-proliferative characteristics in leukemia models compared to **1**.

* DATA NOT INCLUDED IN THE THESIS

GENERATION 8, 9 AND 10 ANALOGS (RESULTS AND DISCUSSION)

Biological Evaluation of Generation 8 Analogs (A78*- A83) via an M2H Luciferase-based System.

Rexinoids can cause a signaling cascade resulting in transcription of RXR target genes that control cellular differentiation, apoptosis, and proliferation, but only after they induce RXR-RXR homodimerization (Panchal and Scarisbrick., 2015). Hence, a mammalian-2-hybrid (M2H) luciferase assay was utilized to ascertain the potency of RXR-RXR homodimerization induced by our analogs compared to bexarotene. In the M2H assay, human U87 glial cells were transiently transfected with the plasmid components of the M2H system (see Methods, Chapter 2), and the cells were subsequently dosed with either ethanol (vehicle), 100 nM bexarotene, or the indicated analog. After 24 hours of ligand exposure, the transcription of the luciferase gene, which is directly proportional to the degree of RXR-RXR homodimerization, was measured via a luminescence assay. Due to differences in structure and chemical properties in our novel generation of analogs, the compounds were separated into "functional" Generation 8 (A78-A83), Generation 9 (A84-A91) and Generation 10 (A92-A100) groupings. In this set of experiments, homodimerization and subsequent transcriptional activity of Generation 8 analogs was compared to bexarotene, which was set to 100%. Transcriptional activity of our analogs ranged from 3.4% to 147.9% of the bexarotene control, with A81 displaying the greatest agonist activity in this cohort of novel analogs (Figure 17A).

*****A78 was grouped with Generation 7 Analogs because its structure was similar to Generation 7 Analogs. A78 M2H and RXRE results are in chapter 4 and A78 LXRE results are in chapter 3.

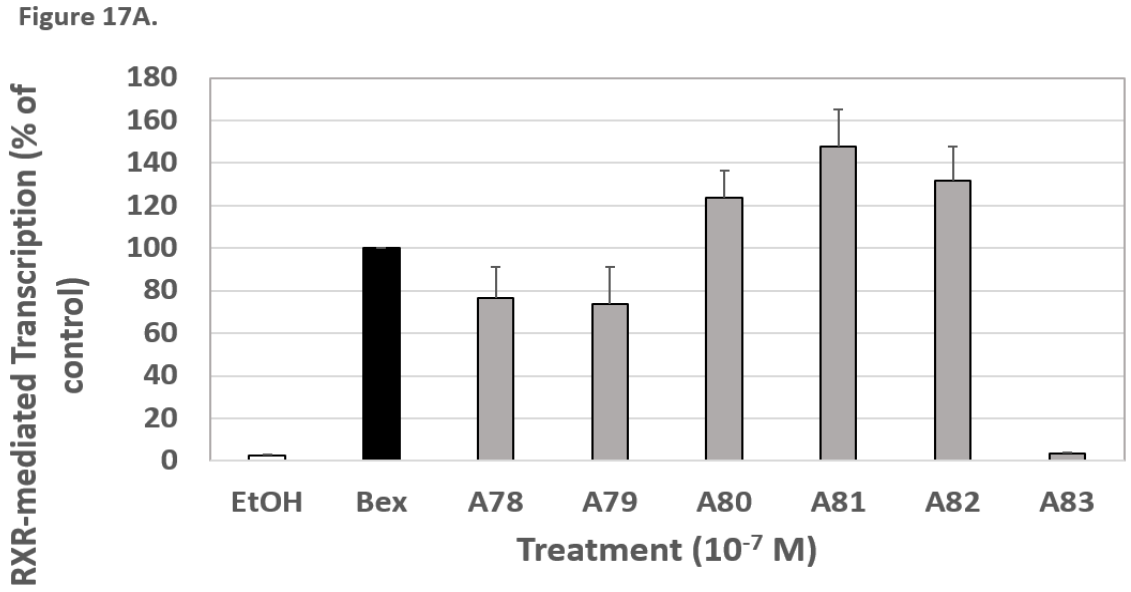

Figure 17. A: Biological Evaluation of Generation 8 (A78-A83) RXR Agonists Via An M2H Luciferase-Based System. Human glial cells (U87) were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Biological Evaluation of Generation 9 Analogs (A84-A91) via an M2H Luciferase-based System.

We evaluated the activity profile of our novel Generation 9 analogs and compared it to bexarotene, which was set to 100% using the same M2H luciferase assay as described above. Transcriptional activity of our analogs ranged from 88% to 168% of the bexarotene control. Analogs A84 and A87 -A91 surpassed bexarotene in terms of their activity (Figure 17B).

Figure 17B.

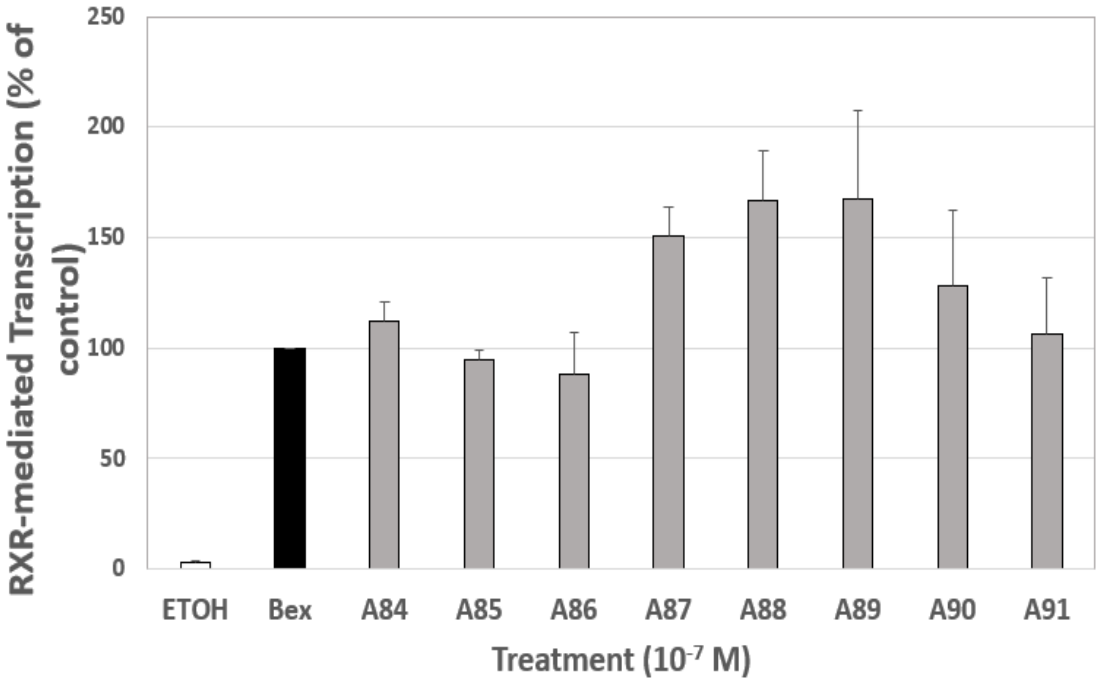

Figure 17. B: Biological Evaluation Of Generation 9 (A84-A91) RXR Agonists Via An M2H Luciferase-Based System. Human glial cells (U87) were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Biological Evaluation of Generation 10 Analogs (A92-A100) via an M2H Luciferase-based System.

RXR-RXR homodimerization capability of our novel Generation 10 bexarotene analogs was assessed by employing M2H luciferase assay as described above. The activity of the

analogs was compared to bexarotene. Transcriptional activity of our analogs ranged from 9% to 128% of the bexarotene control. Analogs A94 and A98 surpassed bexarotene in terms of inducing RXR-RXR homodimer formation (Figure 17C).

Figure 17C.

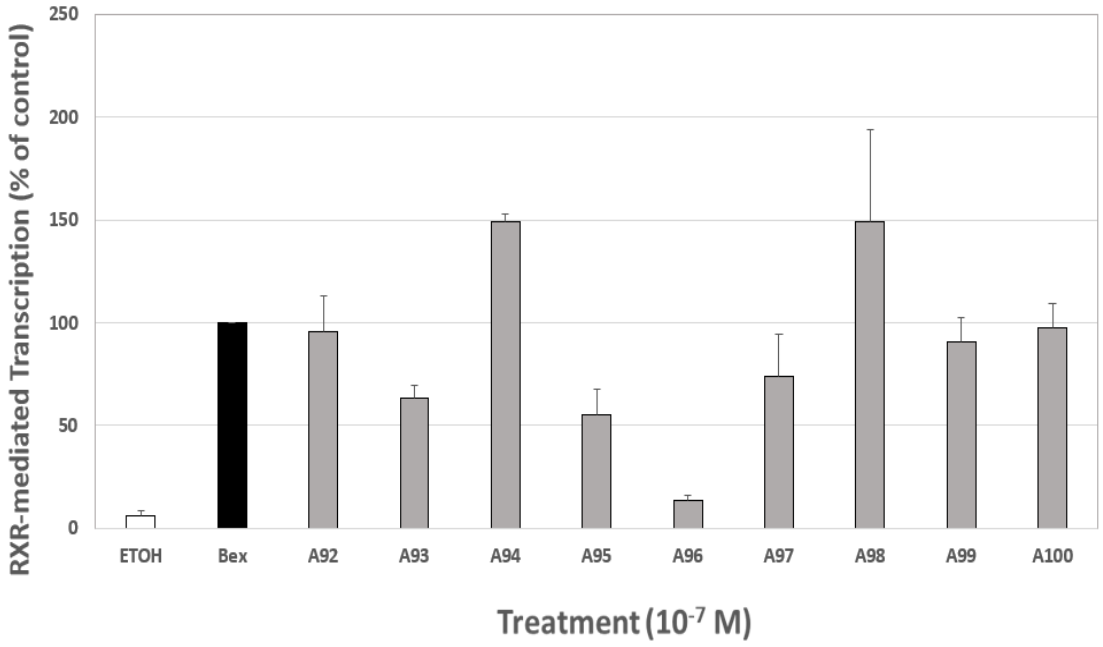

Figure 17. C: Biological Evaluation Of Generation 10 (A92-A100) RXR Agonists Via An M2H Luciferase-Based System. Human glial cells (U87) were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 8 Analogs via an RXRE Luciferase-based System.

Following genesis of the RXR-RXR homodimer, it is requisite for the RXR-RXR homodimer to affiliate with the RXRE in DNA to execute transcriptional regulation. To

assess the capability of our analogs to assist the translocation of the RXR-RXR complex into the nucleus to carry out functions associated with rexinoid signal transduction, we employed an RXRE luciferase assay where transcription of the luciferase gene is directly proportional to RXR-RXR homodimer binding to RXRE. We used human glial U87 cells and transfected them with a plasmid containing an authentic RXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 8 analogs was compared to bexarotene set to 100%. In this set of experiments, transcriptional activity of our analogs ranged from 39.9% to 197.3% of the bexarotene control (Figure 18A), with A82 possessing the highest activity in this cohort. Interestingly, the overall trend seen in the results of the M2H assay very closely mimics what was measured in the RXRE assay. This is an expected and reasonable observation as RXR-RXR homodimerization and homodimer association to the RXRE are consecutive molecular functions necessary for rexinoids to produce their pharmacologic effects.

Figure 18 A.

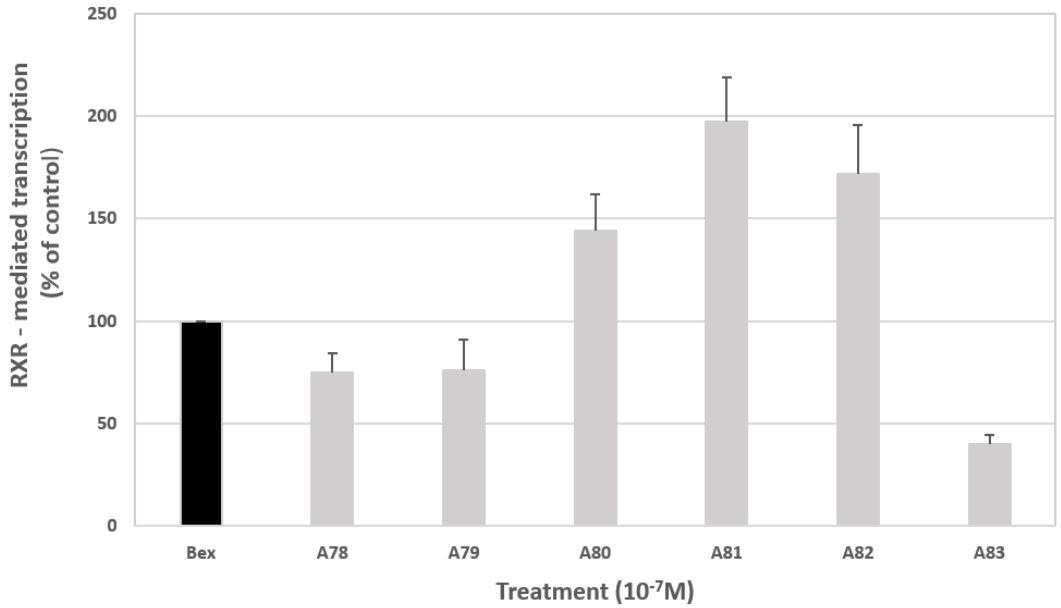

Figure 18. A : Biological Evaluation Of Generation 8 (A78-A83) RXR Agonists Via An RXRE Luciferase-Based System. Human glial cells (U87) were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 9 Analogs via an RXRE Luciferase-based System.

In this parallel set of RXRE experiments, we employed the RXRE assay and compared our Generation 9 analogs to bexarotene. Transcriptional activity of these analogs ranged from 110% to 169% of the bexarotene control (Figure 18B). Analogs A84-A91 all displayed a greater activity trend than bexarotene. Again, the trend in these results closely resemble what was observed in the M2H assay, thus further validating both assay systems.

Figure 18B.

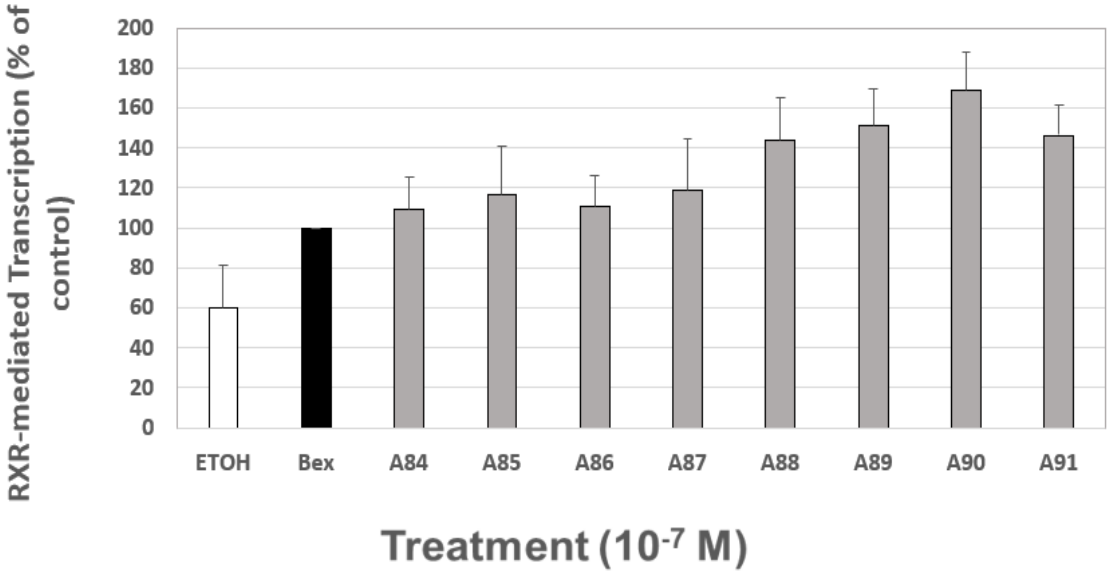

Figure 18. B: **Biological Evaluation Of Generation 9 (A84-A91) RXR Agonists Via An RXRE Luciferase-Based System**. Human glial cells (U87) were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 10 Analogs via an RXRE Luciferase-based System.

In this separate set of RXRE experiments, we performed the RXRE assay and compared our Generation 10 analogs to bexarotene. Transcriptional activity of these analogs ranged from 46.3% to 129.6% of the bexarotene control (Figure 18C). Analogs A92, A94, A98

and A99 displayed a greater mean activity trend than bexarotene.

Figure 18C.

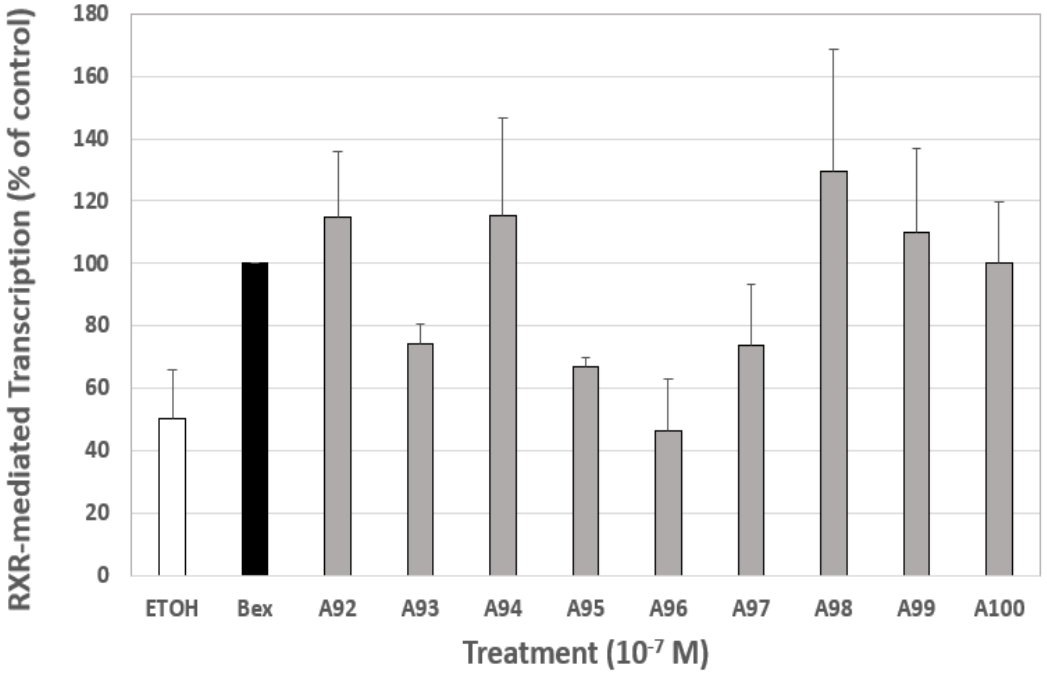

Figure 18. C: Biological Evaluation Of Generation 9 (A84-A91) RXR Agonists Via An RXRE Luciferase-Based System. Human glial cells (U87) were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 8 Analogs via an LXRE Luciferase-based System.

We next tested the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). We

utilized human glial cells (U87) and transfected with a plasmid containing an authentic LXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 8 analogs was compared to bexarotene alone set to 100%. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or synergistic effects of dual ligand activation of the RXR-LXR heterodimer. In this set of experiments, transcriptional activity of our analogs alone ranged from 161.38% to 253.64% of the bexarotene control (Figure 19A). In this cohort, all the analogs alone, or when combined with TO, have better activity than Bexarotene or Bexarotene+TO in activating LXRE.

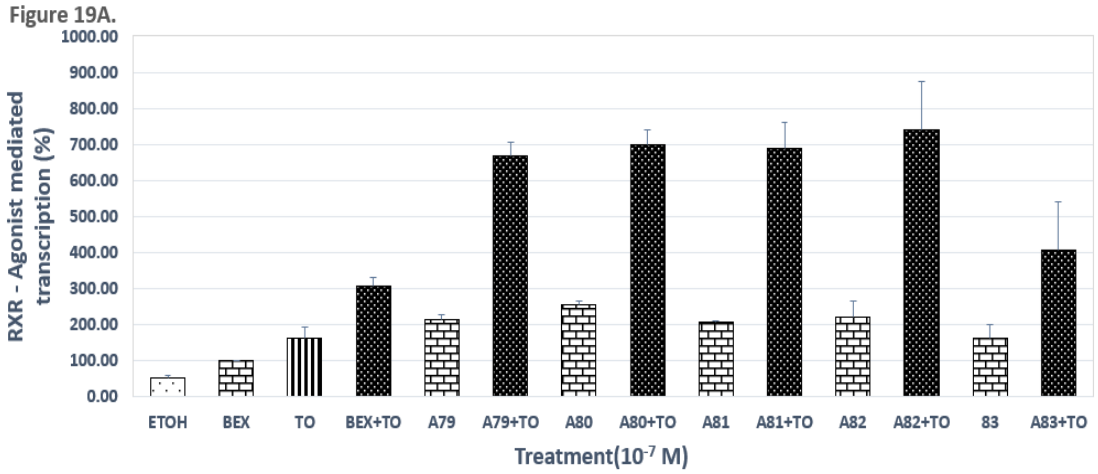

Figure 19. A: Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. Human glial (U87) cells were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to Bexarotene, set to 100%.

Furthermore, it is paramount to consider the activity of the analogs in the context of the RXR-RXR homodimer activity, and to thus "normalize" the LXR/LXRE heterodimer activation in order to yield a LXRE Heterodimer Specificity (LHS) score. The results of this LHS analysis (Figure 19B) revealed that many of our novel compounds in generation 8 (e.g., A79, A80, A82 and A83) possessed greater LXRE/RXRE activity via increased heterodimer specificity than the parent bexarotene.

Figure 19B.

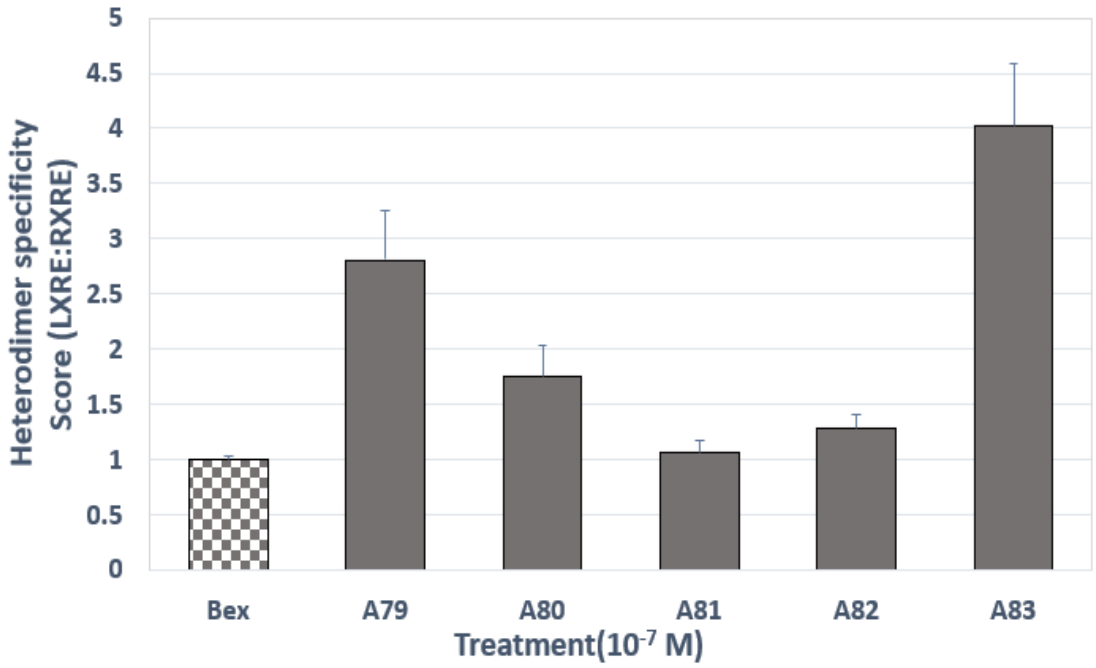

Figure 19. B: Heterodimer Specificity Score -The "Heterodimer Specificity Score" was determined by the LXRE:RXRE ratio with Bexarotene set to 1.0. See text for definition.

Assessment of Generation 9 Analogs via an LXRE Luciferase-based System.

In this cohort of LXRE experiments, we utilized the LXRE assay and compared our Generation 9 analogs to bexarotene. Transcriptional activity of these analogs alone ranged from 77.4% to 114.3% of the bexarotene control (Figure 20A). A84+TO and A86+TO exhibited higher activity than Bex+TO. The results of the LHS analysis revealed that A84 possessed greater LXRE/RXRE activity via increased heterodimer specificity than the parent bexarotene (Figure 20B).

Figure 20A.

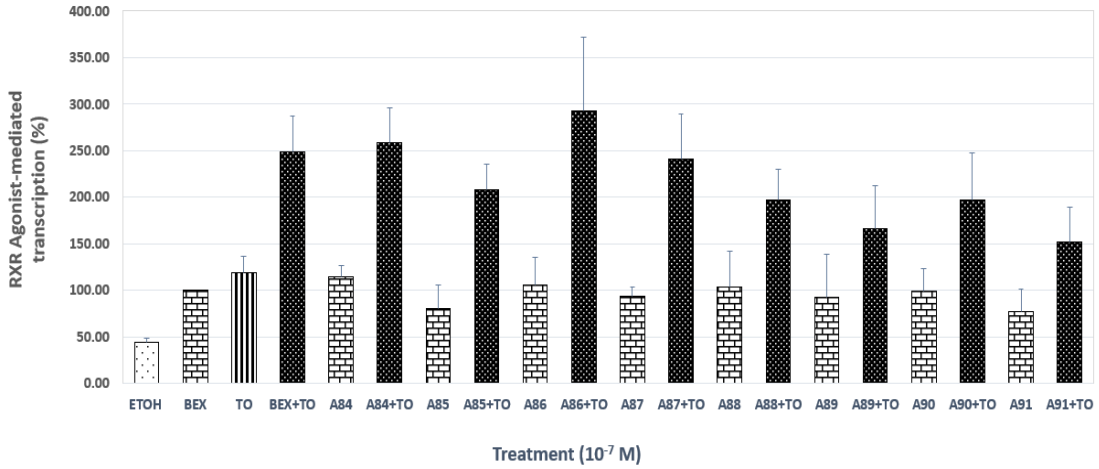

Figure 20. A: Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. Human glial cells (U87) were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to Bexarotene, set to 100%.

Figure 20B.

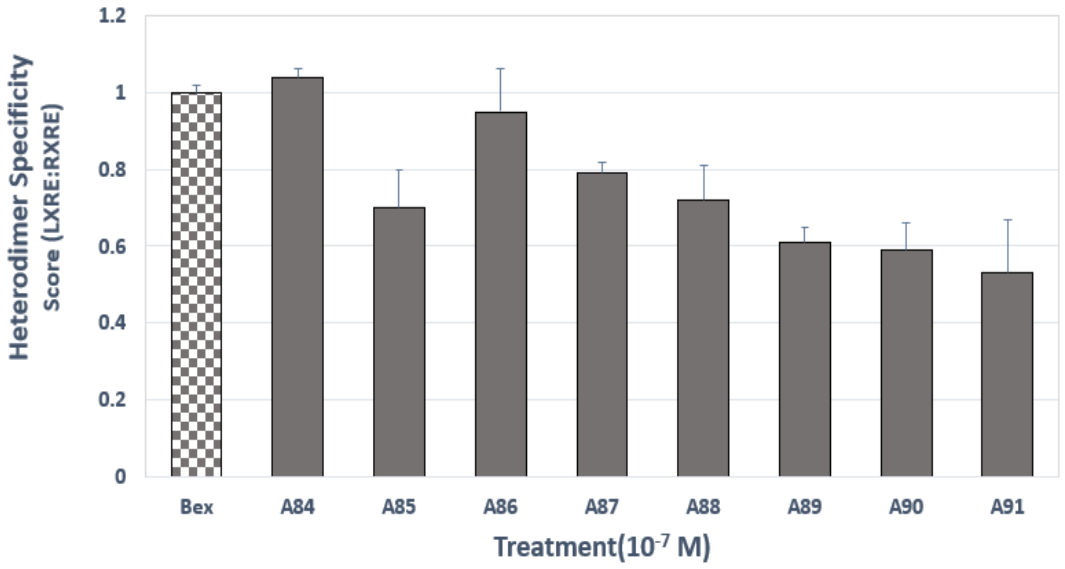

Figure 20. B: Heterodimer Specificity Score - The "Heterodimer Specificity Score" was determined by the LXRE:RXRE ratio with Bexarotene set to 1.0. See text for definition.

Assessment of Generation 10 Analogs via an LXRE Luciferase-based System.

In this set of LXRE experiments, we utilized the LXRE assay and compared our Generation 10 analogs to bexarotene. Transcriptional activity of these analogs alone ranged from 51.5% to 79.6% of the bexarotene control (Figure 21A). There was no remarkable increase in the activity of analogs in combination with the LXR ligand TO901317. The results of the LHS analysis revealed that A96 possessed greater LXRE/RXRE activity via increased heterodimer specificity than the parent bexarotene (Figure 21B).

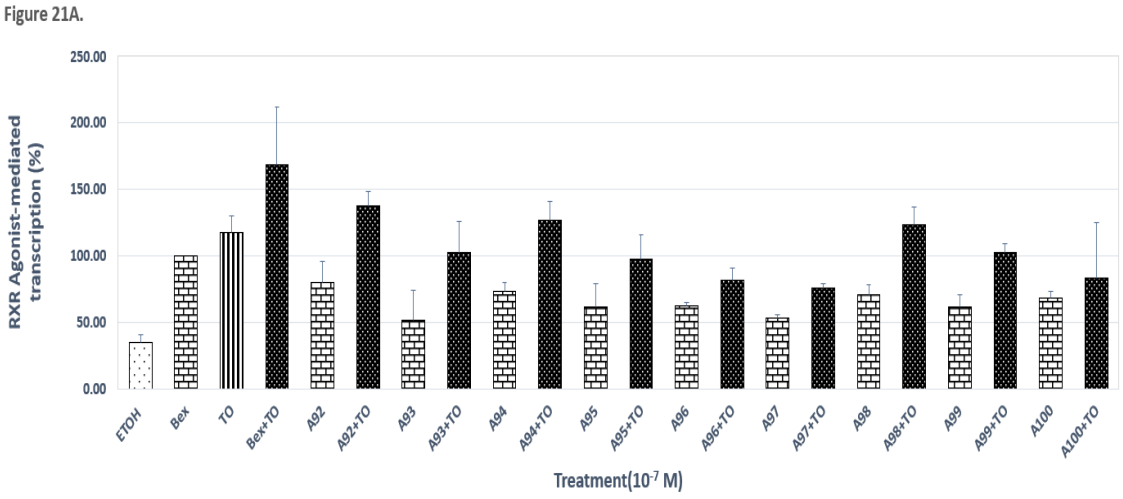

Figure 21. A: Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. Human glial cells (U87) were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to bexarotene, set to 100%.

Figure 21B.

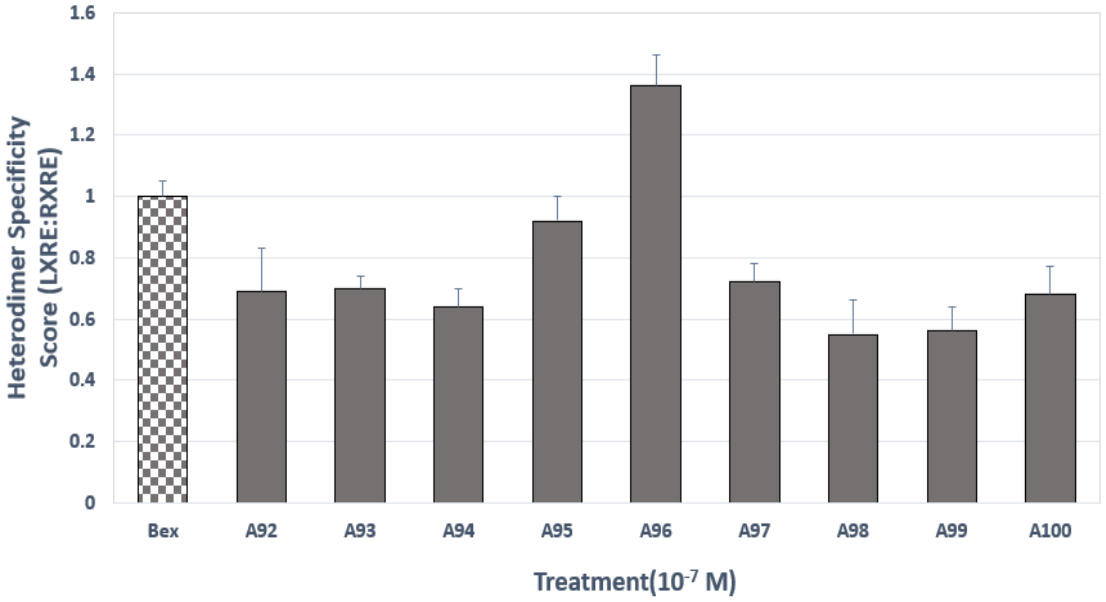

Figure 21. B: Heterodimer Specificity Score - The "Heterodimer Specificity Score" was determined by the LXRE:RXRE ratio with Bexarotene set to 1.0. See text for definition.

Evaluation of Residual RARE activity of Generation 8, 9 and 10 Analogs via RARE Luciferase-based System.

As bexarotene is known to possess "residual" RARE activity, we assessed the ability of Generation 8, 9 and 10 compounds to induce transcription via the retinoic acid response element and retinoic acid receptor (RAR). Human embryonic cells (HEK293) were transfected with human RARα and dosed with 10 nM of either all-trans retinoic acid (RA), the natural ligand for RARα, bexarotene, or analogs. We found that bexarotene possessed and average 37% of the activity of the RA control (Figure 22A, 22B, 22C and 22D). A95 possessed the lowest RARE activity at 4%, which is indistinguishable from the ethanol control while as A89 displayed the greatest RARE

activation at 62% of RA. Other analogs with significant RARE activation greater than bexarotene include A82, A84, A88, and A89 Thus, most of our novel analogs displayed significantly less “cross-over” onto RAR-RARE signaling compared to bexarotene, with 4 exceptions.

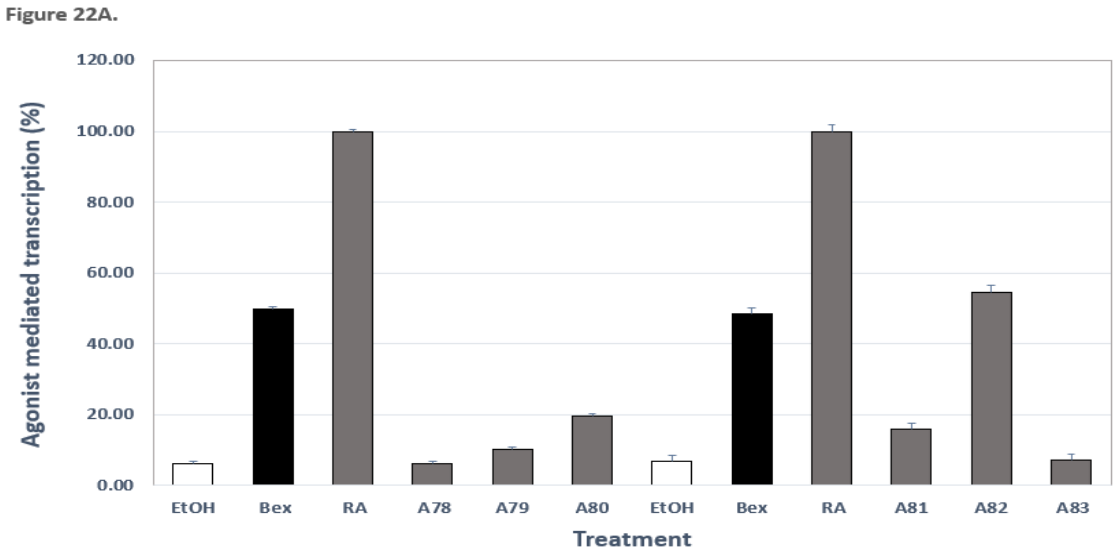

Figure 22. A: Assessment Of RXR Agonists Via An RARE-Luciferase Reporter Based Assay In Human cells.

Figure 22B.

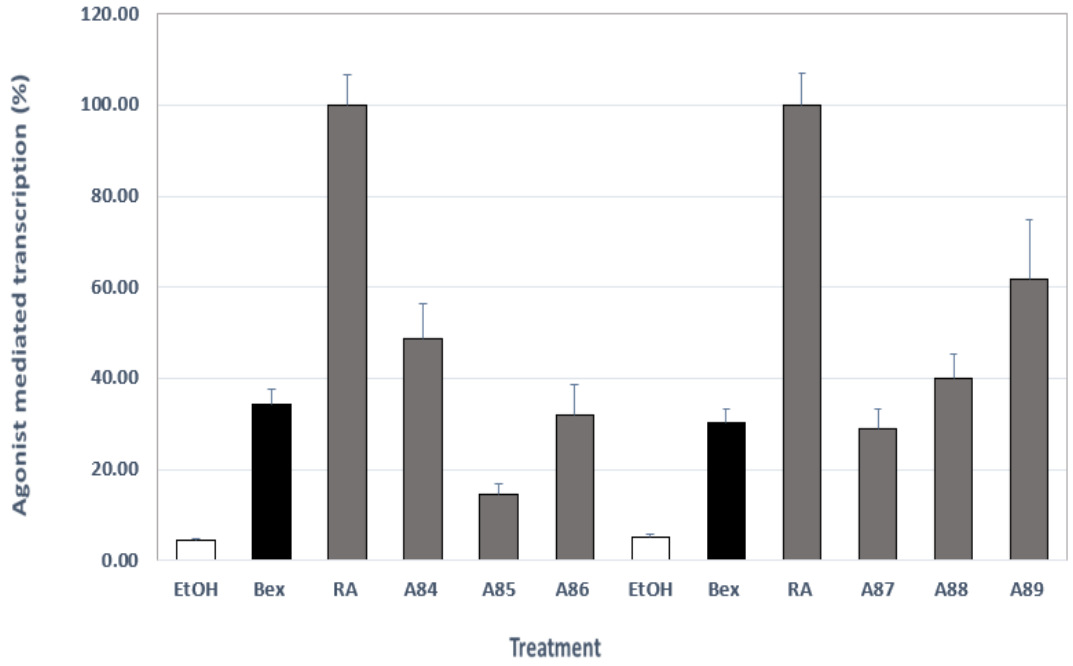

Figure 22. B : Assessment Of RXR Agonists Via An RARE-Luciferase Reporter Based Assay In Human cells.

Figure 22C.

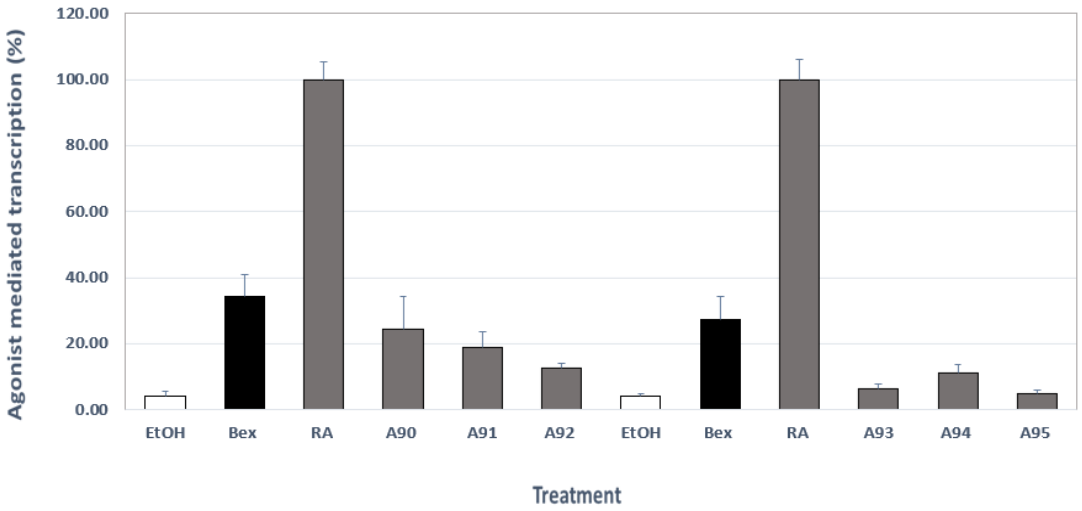

Figure 22. C : Assessment Of RXR Agonists Via An RARE-Luciferase Reporter Based Assay In Human cells.

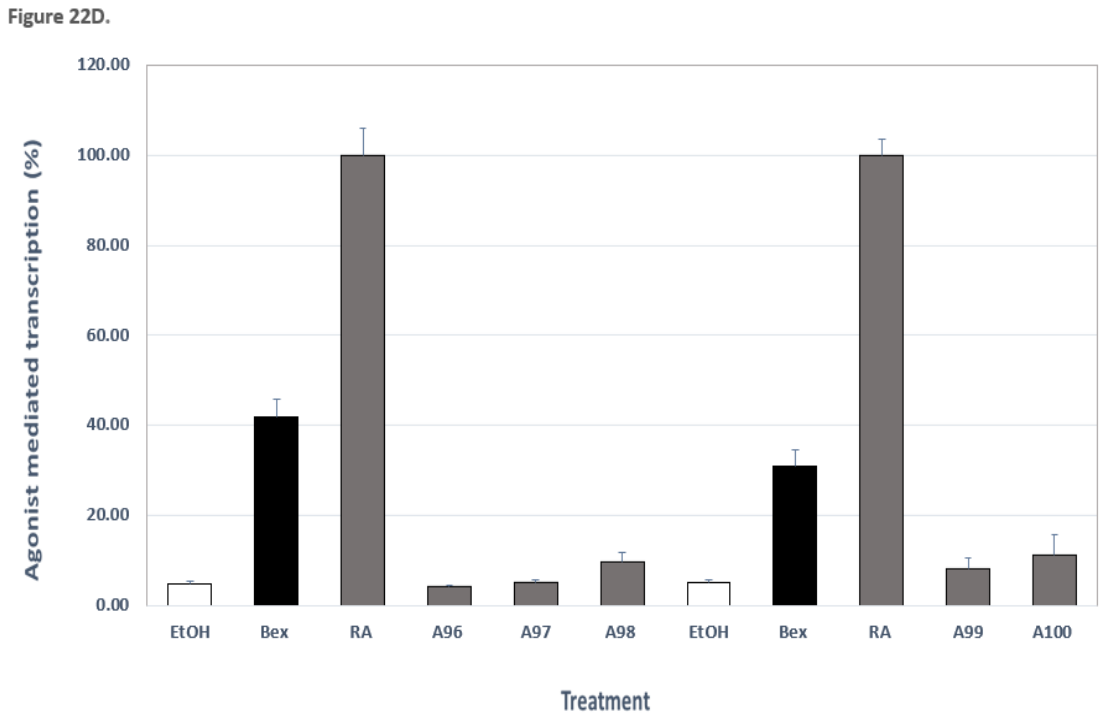

Figure 22. D : Assessment Of RXR Agonists Via An RARE-Luciferase Reporter Based Assay In Human Cells.Figure 22A, 22B, 22C and 22D: Assessment of RXR agonists via a RARE-luciferase reporter based assay in human cells. (A–D) Human embryonic cells (HEK293) were co-transfected with expression vectors for hRXRα, a RARE-luciferase reporter gene, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with bexarotene, analog, or all-trans-retinoic acid (RA) at 10 nM for 24 h.

qPCR Analysis of APOE Gene Induction.

qPCR analysis was performed to determine efficacy of our analogs to upregulate gene transcription of the Apolipoprotein, APOE gene, relative to bexarotene. Considering that not all analogs displayed strong activity in the previous biological assays, only the most potent analogs, namely A80, A81, A82, A84, A86, A92 and A94, were selected for qPCR

evaluation (Figure 23A). In this set of experiments, bexarotene exhibited a mean 6.0-fold increase in APOE gene transcription relative to ethanol control. A80, A81 and A82 yielded low fold inductions, compared to bexarotene, at 4.0-, 3.0- and 3.0-fold, respectively. However, upon combining with LXR ligand TO901317, the fold induction of A80+TO, A81+TO and A82+TO increased to 118-, 65- and 56-fold, respectively. In comparison to Bex+TO, the fold induction of A80+TO was higher than Bex+TO. Therefore, based on these results, which were performed in at least 3 independent biological replicates, A80+TO is more effective than bexarotene in the induction of APOE.

Moreover, in additional qPCR experiments (Figure 23B), the fold induction of A84+TO was 5.78 times higher when compared to Bex+TO which had a fold of 1.0. This means that A84 in combination with the LXR ligand T901317 is 5.78 times more potent than Bex+TO in inducing the expression of APOE. The other analogs in these experiments with TO (A86, A92, A94) all displayed lower APOE induction compared to Bex+TO (Figure 23B).

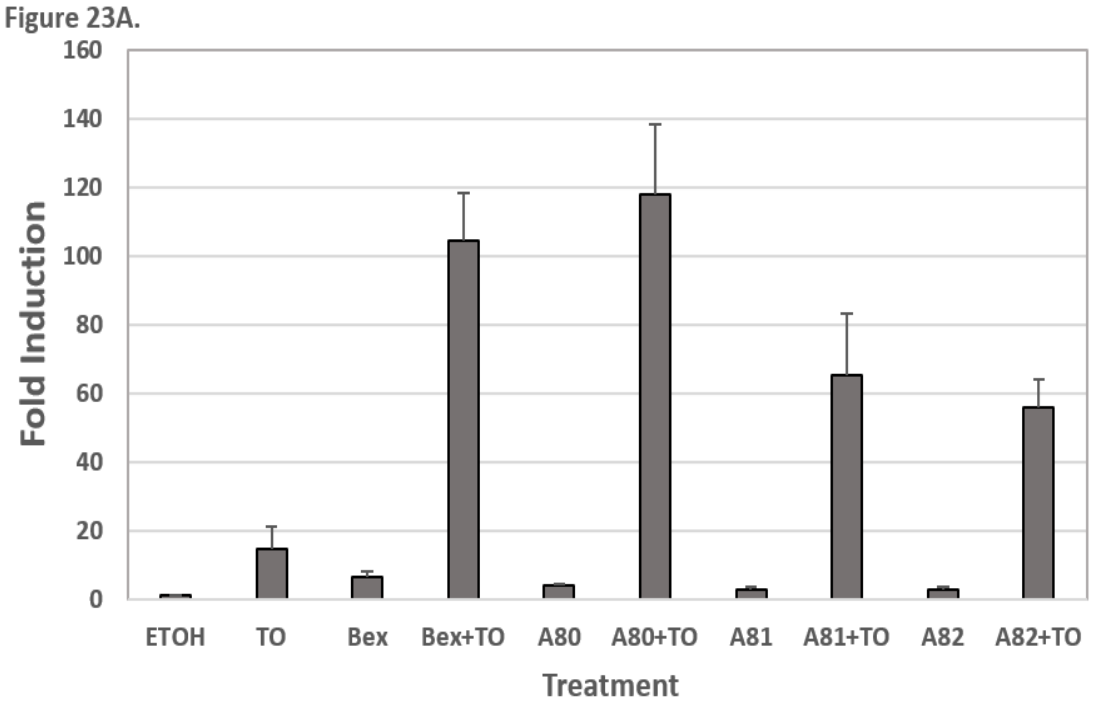

Figure 23. A: qPCR Analysis Of Our Most Potent RXR Agonists (A80-A82) To Induce APOE Gene Expression. Human glial cells were treated for 24 h with bexarotene or the indicated analog at 100 nM. A 0.1 μg portion of DNase-treated RNA was used for cDNA synthesis and subsequent qPCR. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as 2–ΔΔCt. Results are from at least 3 independent biological replicates with multiple replicates in each experiment.

Figure 23B.

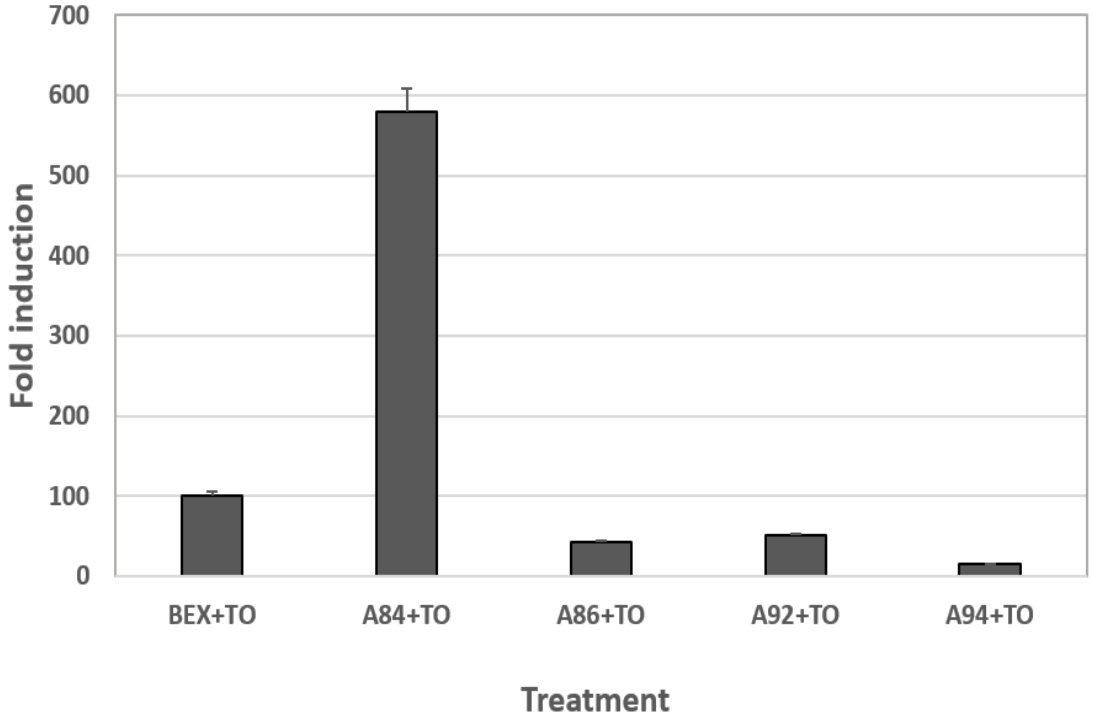

Figure 23. B: qPCR Analysis Of Our Most Potent RXR Agonists (A84, A86, A92, A94) To Induce APOE Gene Expression. Human glial cells (U87) were treated for 24 h with bexarotene or the indicated analog at 100 nM. A 0.1 µg portion of DNase-treated RNA was used for cDNA synthesis and subsequent qPCR. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as 2–ΔΔCt. Results are from at least 3 independent biological replicates with multiple replicates in each experiment.

Discussion

In this study, we describe the evaluation of twenty-two novel analogs of bexarotene using various biological assays to assess RXR specificity and potency. The most robust compounds were also analyzed via qPCR to determine efficacy in the induction of APOE. Our analogs were synthesized as three distinct generations, Generation 8, Generation 9 and Generation 10, possessing specific conserved molecular motifs within their chemical structures. Based on the results obtained in our experiments, most of the Generation 8 analogs demonstrated high selectivity and potency as compared to bexarotene. In the M2H (Figure 17A) and RXRE (Figure 18A) assays, analogs A79-A83 yielded substantially higher RXR-mediated transcription of the luciferase gene than bexarotene, indicating that these analogs are more effective at inducing a response through the RXR-RXRE nuclear signaling pathway. Rexinoids generate their chemotherapeutic effects, at least in part, via RXR-RXRE pathway which enables the transcription of genes involved in cell proliferation, differentiation, and apoptosis. Therefore, analogs A79-A83 are more likely to lead to an improved therapeutic outcome in the treatment of CTCL than bexarotene. Furthermore, some analogs in Generation 9 also performed favorably. In this cohort, more than 50 percent of the analogs yielded either comparable or improved results compared to bexarotene across the M2H and RXRE assays. Most notably, analogs A85-A87 demonstrated an enhanced ability to induce RXR homodimerization (Figure 17B), activate RXRE (Figure 18B). On the other hand, Generation 10 analogs were not as robust in activating RXR-RXRE pathway. We only found a couple of analogs that performed

slightly better than bexarotene in the M2H assays. However, there were four analogs A92, A94, A98 and A99 which had higher activity than bexarotene in the RXRE assays. Hence, we postulate that the potential therapeutic potency of a rexinoid may be predicted based on its performance in these assays and this approach may serve as a practical tool in the assessment of novel rexinoids intended for the treatment of CTCL. However, we still need to test our top performing analogs to determine how well they upregulate the two tumor suppressor genes, ATF3 and EGR3. These genes are involved in tumor suppression pathways and have been previously found to be upregulated in response to retinoids and rexinoids, such as bexarotene (Corona et al.,2016; Wang et al., 2019; Zhang et al., 2015). Our data suggests that although bexarotene may be effective for the treatment of refractory CTCL, introducing new structural motifs to the bexarotene parent compound may in fact yield analogs that are more efficacious and maintain an improved side effect profile due to enhanced selectivity.

We also evaluated the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). LXR has been demonstrated to regulate lipid metabolism and inflammatory responses in the central nervous system, and there is ample evidence that robust cholesterol and lipid metabolism in the brain (including enhanced ApoE expression) are critical to mitigating dementia. Biological evaluation of our novel RXR agonists for their ability to transactivate via an LXRE sequence that is found naturally in the promoter of LXR-RXR controlled

genes including ApoE was carried out in human glial cells (U87) with bexarotene as a comparison. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or synergistic effects of dual ligand activation of the RXR-LXR heterodimer. The results (Figure 19A, Figure 20A and Figure 21A) revealed that in comparison to the parent compound bexarotene alone, single dosing of the cells with any of the tested analogs exhibited more LXR/LXRE activity. Moreover, some of the analogs were found to have increased heterodimer specificity (LXRE/RXRE) as compared to bexarotene indicating that the Generation 8, 9 and 10 analogs have significant potential to activate LXRE-mediated transcription. Most of our novel panel of analogs also possessed less "cross-over" activation than other retinoid pathways including RARE-directed transcription (Figure 22A, 22B, 22C and 22D).

Thus, these analogs not only show promise as the potential therapeutic for CTCL, as but these compounds may also be used in the treatment of Alzheimer's disease due to their ability to significantly and specifically activate the LXR-RXR heterodimer to potentially drive ApoE expression.

REFERENCES FOR CHAPTER 4

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CHAPTER 5

GENERATION 11 ANALOGS (PRELIMINARY RESULTS, DISCUSSION AND CONCLUSION)

Biological Evaluation of Generation 11 Analogs (A101-A126) via an M2H Luciferase-based System.

For a rexinoid to induce transcription of RXR target genes that control cellular differentiation, apoptosis, and proliferation it should be able to induce RXR-RXR homodimerization (Panchal and Scarisbrick., 2015). Therefore, a mammalian-2-hybrid (M2H) luciferase assay was employed to determine the potency of RXR-RXR homodimerization induced by our analogs compared to bexarotene. In the M2H assay, human embryonic kidney (HEK-293) cells were transiently transfected with the plasmid components of the M2H system (see Methods), and the cells were subsequently dosed with either ethanol (vehicle), 100 nM bexarotene, or the indicated analog. After 24 hours of ligand exposure, the transcription of the luciferase gene, which is directly proportional to the degree of RXR-RXR homodimerization, was measured via a luminescence assay. In this set of experiments, homodimerization and subsequent transcriptional activity of Generation 11 analogs (A101-A126) was compared to bexarotene, which was set to 100%. Transcriptional activity of our analogs ranged from 2.3% to 128.3% of the bexarotene control, with A125 and A126 displaying the greatest agonist activity in this cohort of novel analogs (Figure 24).

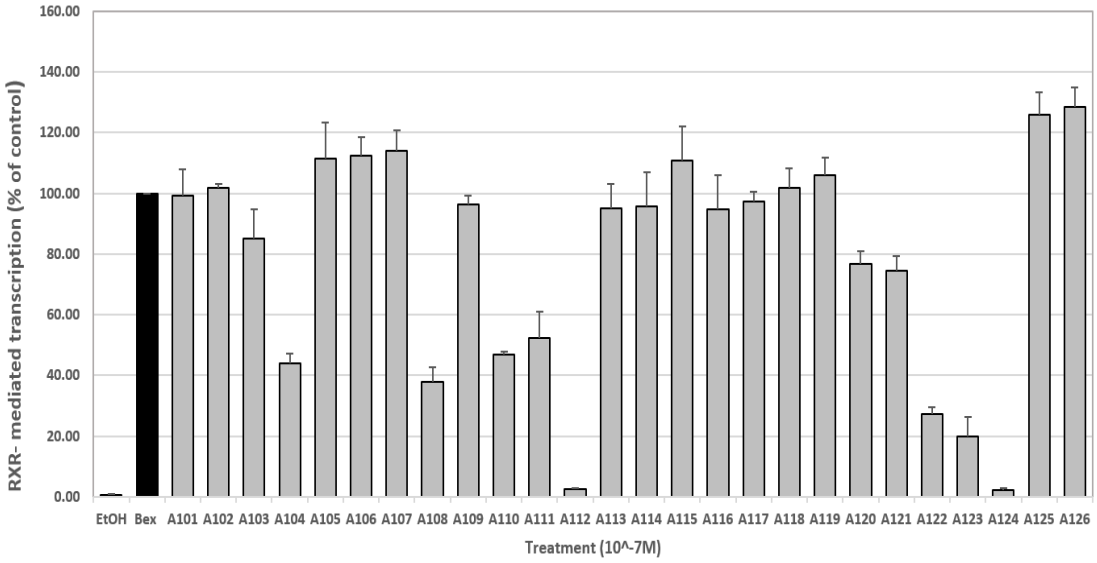

Figure 24: Biological Evaluation Of Generation 11 (A101-A126) RXR Agonists Via An M2H Luciferase-Based System. Human embryonic kidney (HEK-293) cells were co-transfected using a human RXR binding domain (BD) vector (bait), a human RXR activation domain (AD) vector (prey), pFR-Luc, and renilla control plasmids for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-dependent RXR binding and homodimerization, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 11 Analogs via an RXRE Luciferase-based System.

Following the formation of the RXR-RXR homodimer, it is necessary for RXR-RXR to associate with the DNA platform (RXRE) to execute transcriptional regulation. To assess the ability of the analogs to aid the translocation of the RXR-RXR complex into the nucleus to carry out functions associated with rexinoid signal transduction, we employed an RXRE luciferase assay where transcription of the luciferase gene is directly proportional to RXR-RXR homodimer binding to the RXRE. We used human embryonic

kidney (HEK-293) cells and transfected them with a plasmid containing an authentic RXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 11 analogs was compared to bexarotene set to 100%. In this set of experiments, transcriptional activity of our analogs ranged from 16.7% to 147.0% of the bexarotene control (Figure 25), with A126 possessing the highest activity in this cohort. It is an encouraging observation that the results of the M2H assay generally resembles the results of the RXRE assay. This is an expected and reasonable observation as RXR-RXR homodimerization and homodimer association to the RXRE are consecutive molecular functions necessary for rexinoids to produce their pharmacologic effects.

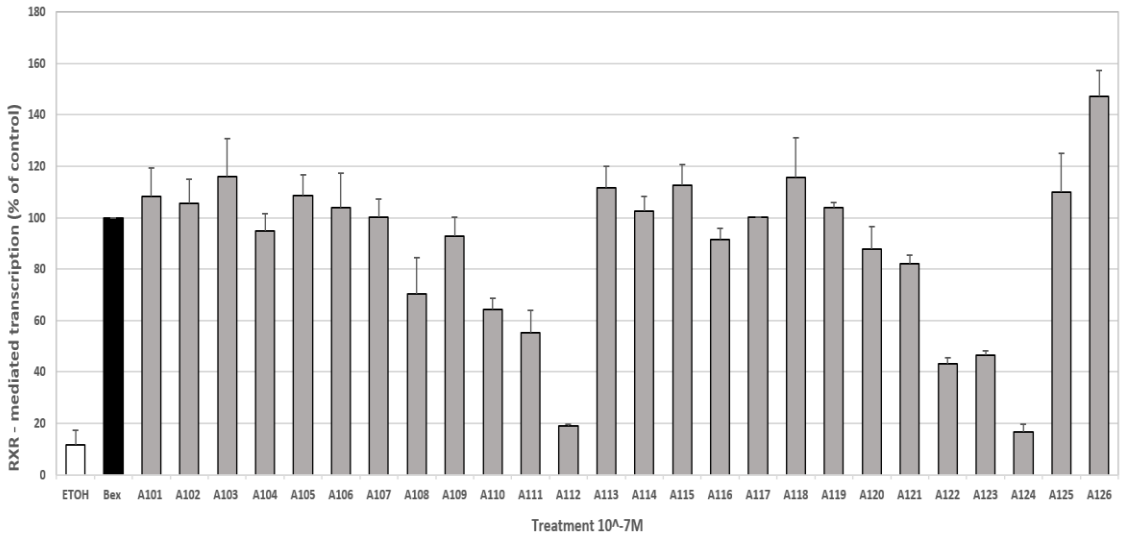

Figure 25: Biological Evaluation Of Generation 11 (A101-A126) RXR Agonists Via An RXRE Luciferase-Based System. Human embryonic kidney (HEK-293) cells were co-transfected using a RXRE luciferase reporter gene, pSG5-human RXRα, and a renilla control plasmid for 24 h utilizing a liposome-mediated transfection protocol. Cells were treated with either the ethanol vehicle or 100 nM of bexarotene or the indicated analog for 24 h. After 24 h the cells were lysed, and a luciferase assay was completed. Analog-

dependent RXR-mediated transcription, as measured by luciferase output, was compared to bexarotene (value set to 100%).

Assessment of Generation 11 Analogs via an LXRE Luciferase-based System.

We next tested the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). We utilized human glial cells (U87) and transfected with a plasmid containing an authentic LXRE DNA sequence upstream of the luciferase gene. Transcriptional activity of Generation 11 analogs was compared to bexarotene set to 100%. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or synergistic effects of dual ligand activation of the RXR-LXR heterodimer. In this set of experiments, transcriptional activity of our analogs ranged from 30.5% to 145% of the bexarotene control (Figure 26). A105, A106, A109, A113, A118, A120 and A122 exhibited higher activity than bexarotene. Upon combination of the analogs with TO, the activity of A107, 108, 113, 117, 118, 119, 120 and 123 was boosted further.

Figure 26.

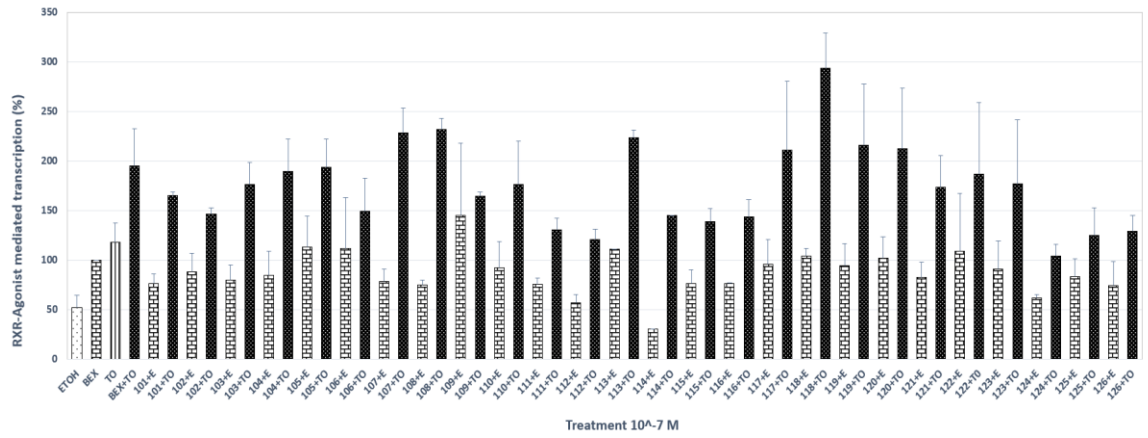

Figure 26: Evaluation Of RXR Agonists To Potentiate LXRE-Mediated Transactivation In The Absence And Presence Of LXR Ligand T0901317. Human glial (U87) cells were transfected with an expression vector for human LXRα, an LXRE-luciferase reporter gene with 3 tandem copies of the LXRE from the human ApoE gene, and a renilla control plasmid. Cells were transfected for 24 hours utilizing a liposome-mediated transfection protocol and then treated with ethanol vehicle, or 100 nM of the indicated compound alone or in combination with 100 nM TO901317 (TO). LXRE-directed activity was compared to bexarotene, set to 100%.

Preliminary qPCR Analysis of APOE Gene Induction.

qPCR analysis was performed to determine the efficacy of our analogs to upregulate gene transcription of the Apolipoprotein E, APOE gene, relative to bexarotene. Considering that not all analogs displayed strong activity in the previous biological assays, only the most potent analogs, namely A101, A105, A107 and A119 were selected for qPCR evaluation (Figure 27). Due to time limitation, we were able to finish the preliminary qPCR analysis of A101, with additional experiments utilizing an expanded set of Generation 11 analogs currently in progress. On comparison with Bex+TO, the fold induction of A101+TO was higher than Bex+TO. A101 exhibited a fold increase of 2.58 compared to BEX+TO which indicates that A101+TO is 2.58 times more potent than

BEX+TO as an inducer of APOE. Therefore, based on these results, which were performed in at least 6 independent biological replicates, A101+TO is more effective than BEX+TO in driving the potent expression of APOE.

Figure 27.

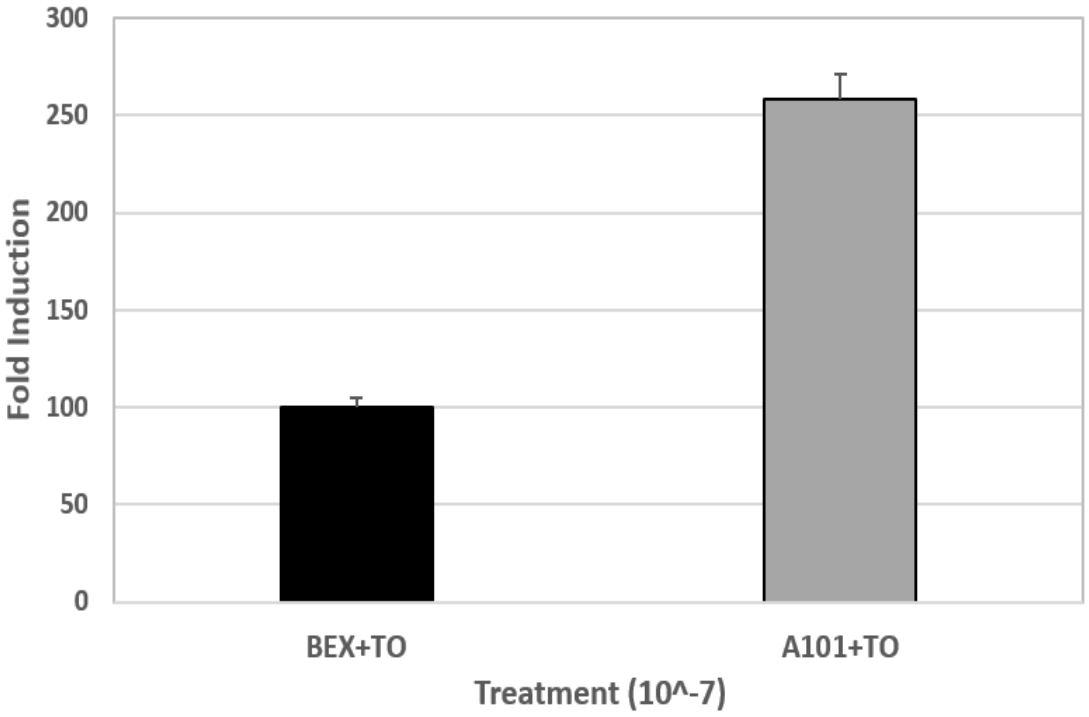

Figure 27: qPCR Analysis Of Our Most Potent RXR Agonists (A101) To Induce APOE Gene Expression. Human glial cells were treated for 24 h with bexarotene or the indicated analog at 100 nM. A 0.1 µg portion of DNase-treated RNA was used for cDNA synthesis and subsequent qPCR. Data analysis was performed using the comparative ΔΔCt method as the means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells) and expressed as 2–ΔΔCt. Results are from at least 6 independent biological replicates with multiple replicates in each experiment.

Discussion

In this study, we describe the evaluation of twenty-six novel analogs of bexarotene using various biological assays to assess RXR potency and specificity. One of the most active analogs (A101) was also analyzed via qPCR to determine efficacy in the induction of APOE. Based on the results obtained in our experiments, most of the Generation 11 analogs demonstrated high selectivity and potency as compared to bexarotene. In the M2H (Figure 24) and RXRE (Figure 25) assays, analogs A101, A102, A105, A106, A107, A115, A118, A119, A125 and A126 exhibited substantially higher RXR-mediated transcription of the luciferase gene than bexarotene, indicating that these analogs are more effective at inducing a response through the RXR-RXRE nuclear signaling pathway. Rexinoids generate their chemotherapeutic effects, at least in part, via the RXR-RXRE pathway which enables the transcription of genes involved in cell proliferation, differentiation, and apoptosis. Therefore, the above analogs are more likely to lead to an improved therapeutic outcome in the treatment of CTCL than bexarotene. In this cohort, about 50 percent of the analogs yielded either comparable or improved activity compared to bexarotene across the M2H and RXRE assays. Most evident, analogs A101, A105-A107, A125 and A126 demonstrated an enhanced ability to induce RXR homodimerization (Figure 24), activate RXRE (Figure 25). Consequently, we conclude that the potential therapeutic potency of a rexinoid may be predicted based on its performance in these assays and this approach may serve as a practical tool in the assessment of novel rexinoids intended for the treatment of CTCL. However, these candidate analogs still need to be assessed in additional assays,

including qPCR to determine their upregulation of tumor suppressor genes, such as ATF3 and EGR3. These genes are involved in tumor suppression pathways and have been previously found to be upregulated in response to retinoids and rexinoids, such as bexarotene (Corona et al., 2016; Wang et al., 2019; Zhang et al., 2015). Our data suggests that although bexarotene may be effective for the treatment of refractory CTCL, introducing new structural motifs to the bexarotene parent compound may in fact yield analogs that are more efficacious and maintain an improved side effect profile due to enhanced selectivity.

We also evaluated the analogs for their ability to bind and activate the liver-X-receptor (LXR) using a liver-X-receptor responsive element (LXRE)-based assay, and we compared the effect in the presence vs. absence of an activating LXR compound (TO901317). LXR has been demonstrated to regulate lipid metabolism and inflammatory responses in the central nervous system, and there is ample evidence that robust cholesterol and lipid metabolism in the brain (including enhanced APOE expression) are critical to mitigating dementia. Biological evaluation of our novel RXR agonists for their ability to transactivate via an LXRE sequence that is found naturally in the promoter of LXR-RXR controlled genes including APOE was carried out in human glial cells (U87) with bexarotene as a control. The activation from this natural LXRE in our system was tested in the presence of either 100 nM RXR agonists alone or in combination with 100 nM of both the RXR agonist and LXR agonist T0901317 (TO). The use of the combination of LXR and RXR agonists was expected to display a more robust response in LXRE transactivation due to additive or

synergistic effects of dual ligand activation of the RXR-LXR heterodimer. The results (Figure 25) revealed that in comparison to the parent compound bexarotene alone, single dosing of the cells with analogs A105, A106, A109, A113, A118, A120 and A122 exhibited more LXR/LXRE activity. Upon combination with TO, analogs A107, 108, 113, 117, 118, 119, 120 and 123 exhibited higher activity than bexarotene indicating that the analogs are more potent than bexarotene when combined with the LXR ligand TO901317 (TO).

Conclusion

Nuclear receptor agonists, specifically bexarotene, are known to have potential therapeutic activity in diseases like cancer and various neurodegenerative disorders such as Alzheimer's and Parkinson's diseases owing to their involvement in transcriptional regulation of genes engaged in vital processes like cell proliferation, development, metabolism, and reproduction and apoptosis (Sever and Glass, 2013). Our research group has previously published work similar to the current study describing the development and biological evaluation of other rexinoids with an emphasis on treating human diseases, such as CTCL and Alzheimer's disease. In 2009, our group evaluated eleven novel analogs of bexarotene, three of which demonstrated similar RXR-mediated transcriptional activity and stimulation of apoptosis in a CTCL system (Wagner at al., 2009). Researchers have recognized RXR and its agonists as potential targets for research and development of therapeutics in cancer and neurodegenerative diseases like Alzheimer's disease because of RXR's function as the special heterodimer partner for approximately one third of all human nuclear receptors (Cramer et al., 2012; Tanaka and

Luca., 2009). The present study builds upon a previous body of research from our rexinoid research group. Furthermore, we continue to demonstrate that minor modifications to the core molecular structure of parent compound bexarotene may lead to the development of novel rexinoids that possess enhanced efficacy and diminished side effect profiles.

Bexarotene is an FDA-approved drug that has been used to treat CTCL with substantial success. However, the side effects of bexarotene therapy are well documented and unfortunately very common, thus bexarotene therapy is combined with a cholesterol lowering medication such as a statin and tetraiodothyronine as thyroid hormone replacement to avoid the side effect of hypothyroidism. Moreover, patients must discontinue the use of bexarotene to allow lab values to normalize as hyperlipidemia and hypertriglyceridemia are risk factors for pancreatitis and cardiovascular disease (McBride, P.E. 2007). These side-effects lead to complications like increased financial burden on the patient thereby reducing compliance to treatment. Due to these reasons, we conclude that the development of novel bexarotene analogs is necessary and should be explored further. Moreover, reprofiling of bexarotene and its novel analogs to treat neurodegenerative disease like Alzheimer's is beneficial because this may save time and financial investment needed for drug design, optimization, and clinical trials since the target and pharmacokinetic profile of bexarotene is well researched. Therefore, evaluating the potential of novel analogs of bexarotene for treatment of AD could prove to be a useful tool to combat AD because the novel analogs have the potential to serve as

inducers of ApoE, thereby enhancing the clearance of Aβ while mitigating the side effects of bexarotene therapy.

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APPENDIX A

PERMISSION TO USE THE DATA

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The data included in the thesis has been published in two manuscripts and a third manuscript is under review. All the co-authors have granted their permission to use the data in the thesis. "Patent applications covering the technologies described in this work have been applied for on behalf of the Arizona Board of Regents."

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