ORPHAN NUCLEAR RECEPTOR 4A1 (NR4A1) AND NR4A2 AS DRUG TARGETS

A Dissertation

by

LEI ZHANG

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Chair of Committee,	Stephen H. Safe
Committee Members,	Timothy D. Phillips
	Qinglei Li
	Natalie M. Johnson
Head of Program,	Ivan Rusyn

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ABSTRACT

NR4A subfamily is a group of orphan nuclear receptors with no known endogenous ligands and includes three members: NR4A1, NR4A2, and NR4A3. NR4A members play important roles in maintaining cellular hemostasis and are involved in multiple diseases. NR4A1 is overexpressed in many inflammatory diseases including solid tumors and is involved in regulation of immune functions. Although an endogenous ligand for NR4A1 has not been identified, several different classes of compounds bind NR4A1 and these include cytosporone B and structurally related analogs, the triterpenoid celastrol and several polyunsaturated fatty acids. Our laboratory has characterized bis-indole derived (CDIM) compounds and multiple polyphenolics as NR4A1 ligands and these compounds are being investigated as novel mechanism-based anticancer agents. This study will take advantage of phytochemical-derived and newly discovered CDIMs compounds that exhibit direct binding to the ligand binding domain of NR4A1 and investigate their role as NR4A1 antagonists (or inverse NR4A1 agonists) that block tumor growth. This study identified natural products such as resveratrol, piperlongumine, quercetin, and kaempferol as NR4A1 ligands with effects similar to that observed for inverse N4AR1 agonists. These natural products show potential as therapeutic strategies for NR4A1-associated diseases. The study found that resveratrol binds NR4A1 with high affinity and acts as an antagonist that inhibits NR4A1-dependent transactivation in lung cancer cells, suggesting that its anticancer activity may be mediated through inhibition of NR4A1. Furthermore, resveratrol acts like an inverse

NR4A1 agonist and modulates NR4A1-dependent genes and pathways, suggesting its potential as a selective receptor modulator. Piperlongumine has also been shown to inactivate NR4A1, thereby enhancing its anticancer activity. Furthermore, flavonoids including quercetin and kaempferol have been shown to suppress endometriosis by modulating NR4A1-mediated pathways. progress. Due to their natural origin, these natural products have the potential to be safe and tolerable therapeutic candidates. Furthermore, CDIM-derived compounds have been identified as novel dual NR4A1/NR4A2 ligands that are expected to inhibit cancer-promoting pathways and provide new avenues for disease therapy. This study will promote the development and application of natural and synthetic NR4A1/2 inverse agonists as precision therapeutics for targeting cancer and endometriosis patients who have high levels of NR4A1/2 expression.

DEDICATION

To my parents and brother for the unweaving love, support, and encouragement especially during the COVID pandemic. I miss and love them so much!

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INTRODUCTION

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1.1.1. Discovery

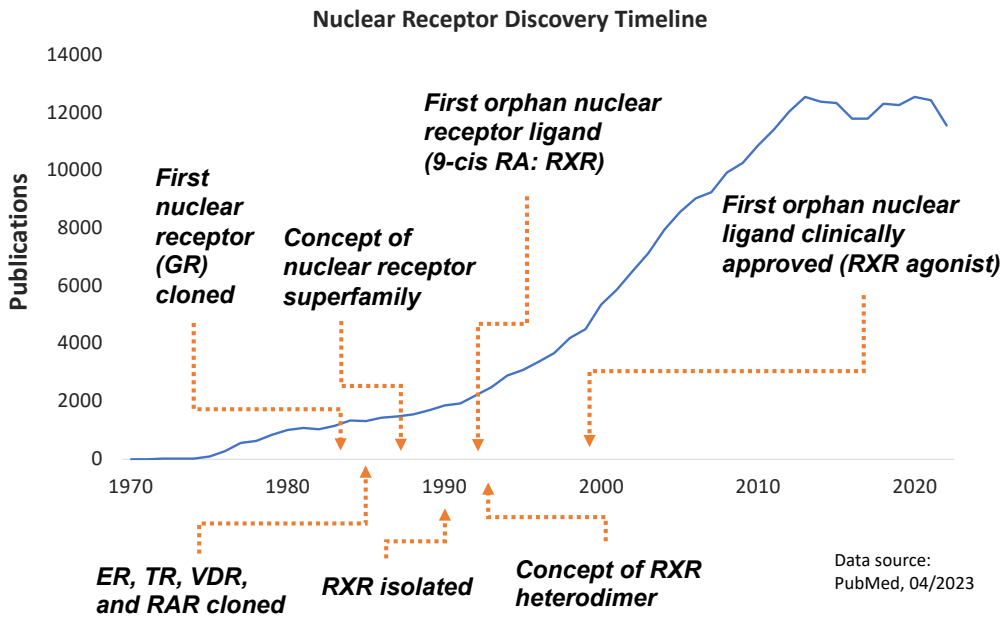


Figure 1-1: Timeline of nuclear receptor discovery.

The dawn of molecular biology in the 1980s led to a series of major breakthroughs in the field. As Figure 1 shows, in 1985, the first complete cDNAs for steroid receptors (glucocorticoid and estrogen receptors) were successfully isolated [1, 2]. The successful isolation and sequencing of the first complete cDNA of a steroid hormone receptor initiated research on nuclear receptors where it was discovered that each gene contains a modular structure that includes DNA binding, ligand binding, and transactivation domains [1, 3, 4]. In the same year, the first full-length nuclear receptor was cloned by

the laboratory of Ronald Evans, namely the human glucocorticoid receptor (GR, encoded by the gene NR3C1) [5]. Around this time, Pierre Chambon's laboratory cloned the first estrogen receptor (ERα, encoded by ESR1) [6]. Studies on the endocrinology and mechanism of action of steroid and thyroid hormones indicated that their action was associated with a receptor in the nucleus. Upon binding to a ligand, this type of receptor-activated transcription of tissue-specific sets of target genes [7, 8]. Nuclear receptors have subsequently been characterized with respect to their ligand binding, interaction with partner proteins, and their binding to cognate cis-elements in target gene promoters. The results show that nuclear receptors primarily bind lipophilic hormones and other small molecules that activate the regulation and expression of specific target genes [9]. Transcriptional regulation by hormone-receptor complexes is a fundamental process embedded in the intracellular signaling pathways of lipophilic endocrine hormones and vitamins [10-12]. Thus, the concept of "Nuclear receptor" has evolved and persisted for several decades.

After initial discoveries of "endocrine receptors", another group of nuclear receptors called orphan receptors were discovered in the 1990s and at the time the identity of their endogenous ligands was unknown. During the study of the retinoid X receptor (RXR), the first endogenous ligand for this receptor, 9-cis retinoic acid (a metabolite of vitamin A) was discovered and shown to bind RXR. Thus, RXR became the first member of the adopted orphan nuclear receptor family [13-15]. The discovery of RXR and its ligand

ushered in a new era in the study of nuclear receptors and their linked ligands and associated signaling pathways, which were dependent on RXR-receptor heterodimers.

One of the major discoveries derived from RXR cloning was that RXR acts as a heterodimeric cis partner with other nuclear receptors including many adopted nuclear receptors [13-15]. The first xenobiotic ligand-receptor heterodimer was discovered in a cotransfection study with an orphan nuclear receptor and a fibrate [16]. Since fibrates promote peroxisome proliferation, this type of orphan nuclear receptor was named peroxisome proliferator-activated receptor (PPAR) which was then identified as a family of receptors that bind the fatty acids [17]. PPARs were the first class of orphan nuclear receptors shown to heterodimerize with RXR [18] and formation of these RXR heterodimers greatly increased the diversity and complexity of receptor binding sites. The discovery of nuclear receptors that responded to xenobiotics resulted in the identification of several receptors that regulated expression of different forms of cytochrome P450 (CYP).

By 1990, 15 nuclear receptor superfamily members had been identified as receptors for lipophilic hormones and other small molecules. Today, the family has grown to more than 150 members, spanning a vast array of animal species from worms to insects to humans. Of the 48 human nuclear receptors endogenous ligands for about 50% of these receptors have not yet been identified. Moreover, like the steroid hormone nuclear receptors, most of the orphan receptors are also potential drug targets. They are potential

as therapeutic targets was predicted when receptors for steroids and thyroid hormones, vitamins A and D, were first discovered. Drugs that target these receptors are among the most widely used and commercially successful pharmaceuticals. For example, Bexarotene and alitretinoin (RXR), fibrates (PPARa), and thiazolidinediones (PPARg) are drugs approved for the treatment of cancer, hyperlipidemia, and type 2 diabetes respectively [19]. Therefore, RXR and its orphan receptor partners have great potential to be relevant as therapeutic targets for many years to come.

1.1.2. Classification of nuclear receptors

A nuclear receptor is a member of a family of proteins that regulate intracellular transcription of specific target genes [20, 21]. These proteins define a superfamily responsible for important aspects of development, differentiation, reproduction, and metabolic homeostasis in eukaryotes. This superfamily is typically divided into three classes, which are illustrated in Figure 2. Class I is the steroid hormone receptor family, which includes the progesterone receptor (PR), estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), and mineralocorticoid receptor (MR). Class II receptors are the thyroid/retinaldehyde family that form RXR heterodimers, and this includes the thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), and peroxisome proliferator-activated receptors (PPARs). The class III receptors are known as the orphan receptor family, which defines a group of proteins identified by comparative sequence analysis as belonging to the nuclear receptor superfamily, but their endogenous ligands are unknown.

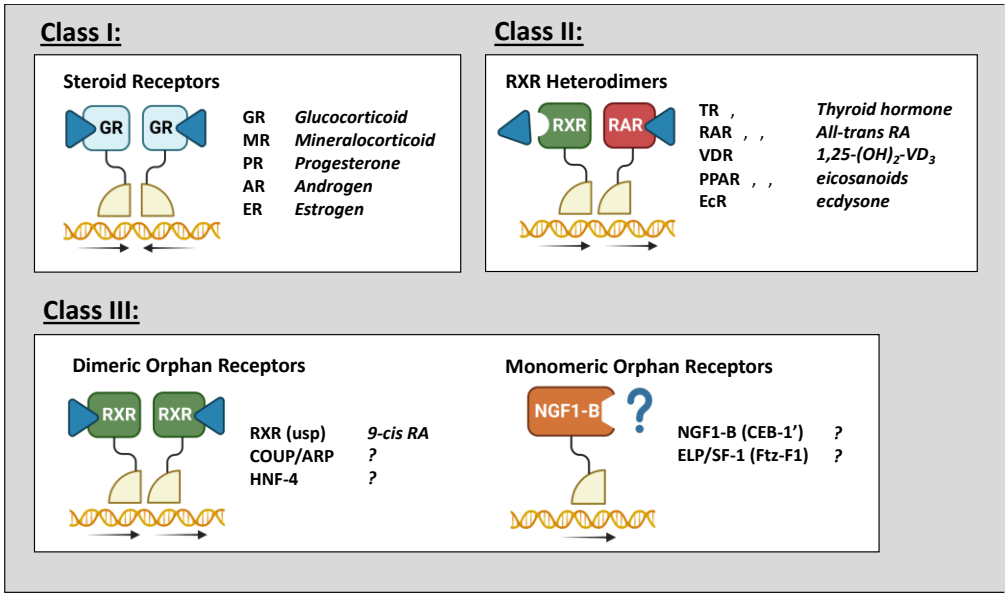


Figure 1-2. The groups of nuclear receptors. Nuclear receptors are grouped into three classes according to their ligand binding and DNA binding: steroid receptors, RXR heterodimers, and orphan receptors. Shown are representative receptors for each group.

Although all nuclear receptors can regulate gene expression, there are subtle differences in the biochemical mechanisms of these receptors within the three classes. The steroid hormone receptors release heat shock proteins and enter the nucleus after binding their hormonal ligand. Once inside the nucleus, the receptors bind as homodimers to imperfect palindromic response elements located at upstream promoter sites. The estrogen receptor (ER) recognizes a hexanucleotide consensus sequence of AGGTCA, while other steroid hormone receptors recognize the consensus AGAACA sequence. DNA binding by the steroid receptors is coupled with the recruitment of coactivator proteins, which then interact with the transcriptional machinery to activate transcription. The steroid hormone receptors interact as head-to-head dimers, where one motif binds to a highly conserved hexanucleotide half-site and the second motif binds to a less

conserved hexanucleotide sequence. The two half-sites are separated by an invariant three nucleotides, although the type of nucleotide is not highly conserved.

Class II nuclear receptors normally form heterodimers where TR, VDR, RAR, and PPAR bind the retinoid X receptor (RXR) to form a heterodimer that interacts with a specific repeat response element. The protomers, or individual subunits, within the heterodimer recognize and bind to a specific six-nucleotide sequence of DNA, which is called a consensus sequence. The consensus sequence recognized by the heterodimer is AGGTCA. Unlike other proteins, such as steroid hormone receptors, which bind to DNA as homodimers, these proteins bind in a head-to-tail orientation [22]. This means that one protomer binds to the DNA and then the other protomer binds to the same DNA molecule adjacent to the first protomer, rather than binding to another DNA molecule as observed for homodimers. These proteins can accommodate small changes in the number of nucleotides between the two halves of the DNA sequence to which they bind. This allows the protein complex to recognize and bind a wider range of DNA sequences, which gives it greater combinatorial specificity. In the presence of agonist ligands. The heterodimers tend to stay bound to their response elements regardless of the presence or absence of agonist ligands (molecules that bind to a receptor and activate a biological response). However, in the absence of agonist ligands, gene activation is prevented by interactions between the DNA-bound heterodimer and corepressor proteins. When an agonist ligand binds the heterodimer, it results in the release of the corepressor proteins and the recruitment of coactivator proteins, which lead to activation of transcription.

Therefore, the binding of agonist ligands to heterodimers is a crucial step in the regulation of gene expression.

Since their ligands have not yet been discovered, the function of orphan nuclear receptors is still not completely understood. Class III nuclear receptors are orphan receptors that can either bind to response elements on their own as monomers or as with RXR heterodimers to carry out their function. Orphan receptors recognize response elements similar to the class II receptors. The DNA sequences surrounding the recognition sites are also crucial for their function because some orphan receptors can also bind as homodimers. Overall, our understanding of how orphan receptors regulate gene expression is still developing and these receptors probably use a combination of hetero and homodimerization binding to carry out their regulatory functions.

Studies on structural alterations within the receptor, coactivating proteins and the promoter DNA are necessary for nuclear receptor activity. Localized adjustments to helical orientation or more extensive disorder-order transitions may be involved in these structural alterations. However, the mechanisms and role of these changes are not well understood. Additionally, nuclear receptor-coactivator interactions on the promoter involve highly unstable and time-dependent processes that are not static and can involve a wide variety of nuclear proteins, which cycle on and off the receptor-DNA-protein complex [23, 24].

1.1.3. Structures and functions

Nuclear receptor structures and functions are intimately related. Early studies showed that nuclear receptor proteins contain two structural subunits: a highly conserved DNA-binding domain (DBD) that is in the middle of the protein and a moderately conserved ligand-binding domain (LBD) that is located at the carboxy-terminal of the protein [25, 26]. Subsequent research identified other domains with multiple subunits that are required for a functional receptor.

1.1.3.1 DNA-binding domain

The DNA-binding domain (DBD) of an NR is adjacent to a short amino acid sequence called the hinge, which connects the DBD to the receptor ligand-binding domain (LBD) (Figure 3). The functional properties of the hinge sequence are not fully understood, but it can be phosphorylated, and phosphorylation has been linked to increased transcriptional activation [27, 28]. Many nuclear receptors contain an amino acid sequence preceding the DBD called AF-1, which is a transcriptional activation function. Compared to the AF-2 sequence embedded in the LBD, the AF-1 sequence shows weak conservation throughout the nuclear receptor superfamily, and this may explain how closely related steroid hormone receptors can differentially bind similar response elements. The AF-1 sequence not only acts as a ligand-independent transcriptional activator but also synergistically carries out functions in combination with AF-2 [29].

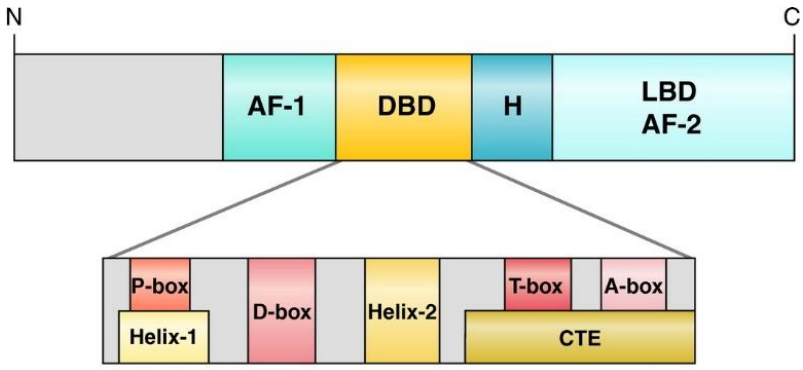


Figure 1-3. Schematic layout of nuclear receptor structure. AF, activation function; DBD, DNA-binding domain; H, hinge; LBD, ligand-binding domain. Shown below is the schematic of the full-length receptor in an expanded view of the DBD, indicating the relative locations of the P-box, D-box, T-box, A-box, helix 1, helix 2, and the C-terminal extension (CTE). Reprinted with permission from [30].

The glucocorticoid receptor (GR) DNA-binding domain is one of the first receptor subunit structures to be determined and is a representative model for the nuclear receptor superfamily [31-33]. The DBD of GR folds into a spherical domain consisting of two non-equivalent zinc finger structures and each zinc atom is coordinated by four cysteine residues. This structure is important for stabilizing domain structure and function because removal of zinc ions results in protein unfolding and loss of DNA-binding activity [34].

RXR-TR heterodimers associated with direct repeats are representative of a class II receptor structure [35]. The overall tertiary fold of each protomer of the heterodimer, including two zinc fingers and an α-helix similar to the GR structure. Furthermore, DNA binding is linked to helix formation and helix ordering occurs in the second zinc finger region and heterodimerization is required [36]. Helical unwinding occurs in the C-

terminal extension (CTE) of the DBD and appears to be necessary for heterodimer formation.

1.1.3.2. Ligand binding domain

The ligand binding domain (LBD) of nuclear receptors plays an important role with multiple functions. First, the LBD provides a unique internal pocket for binding cognate hormones or synthetic ligands. Second, the domain has a transcriptional activation function (AF-2) that is ligand-regulated and required for attracting different coactivator proteins and subsequent interaction with the general transcriptional activation machinery as well as chromatin remodeling proteins [37]. And finally, for high-affinity DNA response element binding, the LBD serves as the primary mediator of solution self-assembly processes (such as dimerization or tetramerization) [38].

In 1995, the high-resolution structure of the retinoid X receptor was reported [39]. The LBD is a compact domain containing 12 alpha-helices, which are arranged in three antiparallel helical sheets. The ligand binding pocket is located inside the LBD structure and is formed by a subset of surrounding helices. The strength and specificity of the LBD-ligand complex are determined by several factors, including hydrophobic interactions, extensive hydrogen-bonding networks, and the size and shape of the binding pocket (rev in. [30]).

The LBD contains a ligand-dependent activation function (AF-2) that can recruit proteins such as the steroid receptor coactivator (SRC) family [37]. This function is accomplished by interactions with a hydrophobic groove that contains several helices including helix 12 (also known as the AF-2 helix) with coactivators containing helical LXXLL motifs that form hydrophobic interactions in the groove. Receptor agonists modulate LBD-coactivator interactions by altering the conformational flexibility of the LBD and helix 12 [39]. In the absence of ligand, the LBD is inactive, either because helix 12 is positioned away from the LBD core structure, or because the domain exhibits a broad repertoire of conformations [40]. Upon ligand binding, helix 12 is stabilized on the LBD surface, allowing formation of a hydrophobic binding groove and recruitment of coactivators [41]. Antagonists and core inhibitors prevent the recruitment of coactivators by inhibiting the ability of helix 12 to approach the core LBD structure [42]. Inhibitors induce helix 12 to bind in the hydrophobic groove without coactivator action [43] or form a long triple-turn helix to prevent helix 12 from acquiring an active conformation and formation of a hydrophobic binding cleft [44]. The antagonist-core inhibitory complex can be further stabilized by the presence of an antagonist, which prevents helix 12 from forming an active conformation and creates a larger binding surface for the core inhibitory protein LXXLL motif [45].

The structure of the nuclear receptor LBD is highly conserved and contains an activating domain that is dependent on the presence of ligands and enables signal transduction and regulation of gene expression through the binding of coactivators and core repressor

proteins. Knowledge of LBD ligands is an important foundation for the development of new drug therapies.

1.1.4. Ligands

Classical endocrinology studies have identified key factors such as thyroid hormones, steroid hormones, vitamins A and D, which bind with high affinity to their cognate receptors. In contrast, reverse endocrinology has also identified low-affinity endogenous ligands derived from dietary lipids [46]. The primary role of the receptors for lipid-derived ligands is to maintain the homeostatic levels of the ligands themselves. Thus, the identified natural ligands turn out to be predictive markers of physiological pathways regulated by their corresponding receptors. Table 1 lists the mammalian nuclear receptors and their ligands.

Table 1-1. Mammalian Nuclear receptors and their ligands. Reprinted with permission from [47].

Common Name	Common Abbreviation	Unified Nomenclature	Ligands
Androgen receptor	AR	NR3C4	androgens
Constitutive androstane receptor	CAR	NR1I3	xenobiotics
Chicken ovalbumin upstream promoter-transcription factor α	COUP-TFα	NR2F1	
Chicken ovalbumin upstream promoter-transcription factor β	COUP-TFβ	NR2F2	
Chicken ovalbumin upstream promoter-transcription factor γ	COUP-TFγ	NR2F6	
Dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1	DAX-1	NR0B1	
Estrogen receptor α	ERα	NR3A1	estrogens
Estrogen receptor β	ERβ	NR3A2	estrogens
Estrogen related receptor α	ERRα	NR3B1	
Estrogen related receptor β	ERRβ	NR3B2	
Estrogen related receptor γ	ERRγ	NR3B3	
Farnesoid X receptor α	FXRα	NR1H4	bile acids
Farnesoid X receptor β[a]	FXRβ	NR1H5	

Table 1-1 continued.

Germ cell nuclear factor	GCNF	NR6A1	
Glucocorticoid receptor	GR	NR3CI	glucocorticoids
Hepatocyte nuclear factor 4 α	HNF4α	NR2A1	[fatty acids]
Hepatocyte nuclear factor 4 γ	HNF4γ	NR2A2	[fatty acids]
Liver receptor homolog-1	LRH-1	NR5A2	[phospholipids]
Liver X receptor α	LXRα	NR1H3	oxysterols
Liver X receptor β	LXRβ	NR1H2	oxysterols
Mineralocorticoid receptor	MR	NR3C2	mineralocorticoids and glucocorticoids
Nerve-growth-factor-induced gene β	NGF1-B	NR4A1	
Neuron-derived orphan receptor 1	NOR-1	NR4A3	
Nur-related factor 1	NURR1	NR4A2	
Photoreceptor-cell-specific nuclear receptor	PNR	NR2E3	
Peroxisome proliferator-activated receptor α	PPARα	NR1C1	fatty acids
Peroxisome proliferator-activated receptor β/δ	PPARβ/δ	NR1C2	fatty acids
Peroxisome proliferator-activated receptor γ	PPARγ	NR1C3	fatty acids
Progesterone receptor	PR	NR3C3	progesterone
Pregnane X receptor	PXR	NR1I2	endobiotics and xenobiotics
Retinoic acid receptor α	RARα	NR1B1	retinoic acids
Retinoic acid receptor β	RARβ	NR1B2	retinoic acids
Retinoic acid receptor γ	RARγ	NR1B3	retinoic acids
Reverse-Erb α	REV-ERBα	NR1D1	[heme]
Reverse-Erb β	REV-ERBβ	NR1D2	[heme]
RAR-related orphan receptor α	RORα	NR1F1	[sterols]
RAR-related orphan receptor β	RORβ	NR1F2	[sterols]
RAR-related orphan receptor γ	RORγ	NR1F3	[sterols]
Retinoid X receptor α	RXRα	NR2B1	9-cis retinoic acid and docosahexanoic acid
Retinoid X receptor β	RXRβ	NR2B2	9-cis retinoic acid and docosahexanoic acid
Retinoid X receptor γ	RXRγ	NR2B3	9-cis retinoic acid and docosahexanoic acid
Steroidogenic factor 1	SF-1	NR5A1	[phospholipids]
Short heterodimeric partner	SHP	NR0B2	
Tailless homolog orphan receptor	TLX	NR2E1	
Testicular orphan receptor 2	TR2	NR2C1	
Testicular orphan receptor 4	TR4	NR2C2	
Thyroid hormone receptor α	TRα	NR1A1	thyroid hormones
Thyroid hormone receptor β	TRβ	NR1A2	thyroid hormones
Vitamin D receptor	VDR	NR1I1	1a,25-dihydroxyvitamin D_3 and lithocholic acid
Ligands in brackets indicate atypical ligands that, by structural studies, appear to be constitutively bound to their receptors. [a]FXRb exists only as a pseudogene in humans [47].			

1.1.4.1 Endocrine receptor ligands

The initial discovery of the glucocorticoid receptor, estrogen receptor, progestin receptor, and vitamin D receptor was based on their ability to bind their corresponding endogenous ligands [9]. Most of the remaining nuclear receptors were characterized either by cloning or by sequence similarity to the hormone and ligands that bind these receptors were subsequently identified. Even the retinoic acid receptor RAR (NR1B1) was originally characterized as an orphan receptor and by the end of the original paper describing its cloning, retinoic acid had been identified as its ligand [3]. Progestins, estrogens, and glucocorticoids can be considered endogenous receptor ligands, derived from cholesterol in the ovary and adrenal cortex, respectively. Likewise, thyroid hormones are produced by the thyroid gland, and vitamin D3 is made in the skin when the sun's ultraviolet rays activate 7-dehydrocholesterol. Thus, the initially identified hormone receptor ligands exhibit not only high receptor binding affinities but are also produced and secreted from the organ in an endocrine manner.

1.1.4.2. Orphan nuclear receptors

After the existence of nuclear receptors was widely recognized, biochemical and cloning experiments were used to reveal their common structure and mode of action. Realizing that nuclear receptors have extensive homology, low-stringency screening of cDNA libraries was used to find new members. This led to the unexpected discovery that some ligands, such as T3 and atRA, regulate development and physiology through multiple receptors [48, 49]. Additionally, multiple cDNAs encoding proteins with structural

features of nuclear receptors were isolated. However, since ligands could not be linked to these receptors based on structural studies alone, they were referred to as "orphan nuclear receptors". It was subsequently determined that there are about a dozen different nuclear receptor-like proteins that was ultimately named orphan receptors [50]. Since then, the study of orphan nuclear receptors and the critical roles of their ligands in development, homeostasis and disease have been characterized. The activity of nuclear receptors is modulated by direct binding of natural and synthetic compounds, making orphan receptors important targets for drug discovery. Recent advances in this field suggest that orphan receptors ligands can be used to treat a variety of diseases, including diabetes, disorders of lipid metabolism and cancer. Even if there is no known natural ligand, the exploration of drugs that target orphan nuclear receptors creates more opportunities for receptor ligand-dependent treatment of diseases and the development of new drugs.

Nomenclature

The nomenclature defining orphan nuclear receptors is arbitrary and often resulted in unrelated nomenclature for the same receptor cloned from different species. The new nomenclature is based on a similar system used for the cytochrome P450 superfamily; Arabic numerals are used to designate gene subfamilies, uppercase letters designate groups, and a second set of Arabic numerals designate individual genes. Receptor subtypes arising from the same gene are identified with lower case letters at the end of the name.

Table 2 lists the known vertebrate orphan nuclear receptors arranged into seven groups (0 to VI) based on molecular phylogenetic analysis. Each group is divided into families by its most commonly used trivial name, with a Greek letter identifying each family member. Receptor subtypes arising from a single gene are identified by Arabic numerals. Each receptor is then identified by its official name and a list of other known trivial names is provided. In this article, orphan nuclear receptors will be referred to by their family name (the most commonly used trivial name) and subtypes will be referred to by their Greek letters.

Table 1-2. Groups of orphan nuclear receptors. Reprinted with permission from [51].

Groups	Families	Subtypes	Isoforms	Nomenclature	Trivial names	Species[a]
I	PPAR	α		NR1C1	PPARα	h, m, r, l, g, x
		β		NR1C2	PPARβ, PPAR6, NUC1, FAAR	h, m, r, l, x
		γ	1, 2	NR1C3	PPARγ	h, k, b, p, m, r, l, x
	Rev-Erb	α		NR1D1	RevErbAα, EAR-1	h, r
		β		NR1D2	RVR, RevErbAβ, BD73, HZF2	h, m, r, c
	ROR	α	1, 2, 3, 4	NR1F1	RORα, RZRα	h, m
		β		NR1F2	RORβ, RZRβ	m, r, c
		γ	1, 2	NR1F3	RORγ, TOR	h, m
	LXR	α		NR1H3	LXRα, RLD1	h, r
		β		NR1H2	LXRβ, UR, NER, RIP15, OR1	h, m, r
	FXR			NR1H4	FXR, RIP14, HRR1	h, m, r
	PXR		1, 2	NR1I2	PXR.1, PXR.2, SXR, ONR1, xOR6, BXR	h, m, x
	CAR	α		NR1I3	hCAR1, MB67	h
		β		NR1I4	mCAR1	m
II	HNF4	α		NR2A1	HNF4	h, m, r, x
		β		NR2A3	HNF4β	x
		γ		NR2A2	HNF4γ	h
	RXR	α		NR2B1	RXRα	h, m, c, x, f
		β		NR2B2	RXRβ, H2RIIBP	h, m, r, x, f
		γ	1, 2	NR2B3	RXRγ	h, m, r, x, c, f

	TR2	α		NR2C1	TR2, TR2-11, xDOR2, aDOR1	h, m, x, a

Table 1-2 continued.

		β		NR2C2	TR4, TAK1, TR2R1	h, m, r
	TLX			NR2E1	T1x, TLL, xTLL	h, m, c, x, f
	COUP-TF	α		NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44	h, m, r, x, f
		β		NR2F2	COUP-TFII, COUPTFB ARP1, SVP40	h, m, r, c, x, f
		γ		NR2F4	xCOUP-TFIII, COUP-TFγ	x
				NR2F5	SVP46	f
				NR2F6	EAR2	h, m,r
III	ERR	α		NR3B1	ERRα, ERR1	h, m
		β		NR3B2	ERRβ, ERR2	h, m, r
		γ		NR3B3	ERRγ	h
IV	NGFI-B	α		NR4A1	NGFI-B, NUR77, N10, TR3,	h, d, r, m, x
		β		NR4A2	NURR1, NOT, RNR1, HZF-3, TINUR, TR3β	h, m, r
		γ		NR4A3	NOR-1, MINOR, TEC, CHN	h, m, r
V	FTZ-F1	α	ELP1, 2, 3	NR5A1	FTZ-F1, SF1, ELP, AD4BP	h, b, m, r, c
		β		NR5A2	FTF, LRH1, PHR1, CPF, FFLR, FF1rA	h, m, c, x, f
VI	GCNF			NR6A1	GCNF, RTR	h, m, x
0	DAX			NR0B1	DAX1, AHCH	h, p, m, r
	SHP			NR0B2	SHP	h, m, r

[a] h, Human; k, monkey; b, bovine; p, pig; l, rabbit; d, dog; m, mouse; r, rat; g, guinea pig; c, chicken; f, fish; a, axololt; x, *Xenopus laevis* [51].

Structural and functional diversity

Most orphan nuclear receptors have the same functional domains as classical nuclear receptors, including a DNA-binding domain (DBD), a ligand-binding domain (LBD), and activation domains (AF1+AF2). However, some orphan nuclear receptors may have shortened regulator domains or they may lack AF-1 or AF-2. Some members of the nuclear receptor superfamily may have a conserved DBD or LBD, but not necessarily both domains in the same molecule. Both DAX-1 and SHP lack nuclear receptor-like

DBDs, whereas numerous nuclear receptor-like gene products encoded by the Drosophila EGON, KNIRPS and KNRL and Caenorhabditis elegans genomes do not have homology with the LBDs of other nuclear receptors [51]. It has been shown that DAX-1 can bind hairpin loop structures in DNA through its unique amino-terminal domain [52],while other intracellular receptors (such as the aryl hydrocarbon receptor) and serum and cell-binding proteins (such as Retinol-binding protein, intracellular retinoic acid-binding protein) bind small lipophilic ligands using a structure unrelated to the nuclear receptor LBD. However, they can still use unrelated domains to bind DNA or ligands.

Except for DAX-1, orphan nuclear receptors recognize specific hormone response elements (HREs) through their DBDs similar to classical nuclear receptors [53, 54]. Many nuclear receptors were found to bind DNA as homo or heterodimers [55]; whereas some orphan nuclear receptors bind DNA as monomers with high-affinity [51]. Monomeric nuclear receptors recognize distinct A/T-rich sequences upstream of a single core half-site using a common CTE (cofactor recognition element) [56-58]. Different N-terminal domains contained within the orphan nuclear receptor ROR (RAR-related orphan nuclear receptor) have been shown to interact with a common CTE to modulate the receptor's binding site specificity [59].

A highly recognizable LBD is present in all vertebrate orphan nuclear receptors and this is often interpreted as their intrinsic ability to bind specific ligands. However, the LBD

may only be necessary for activities such as dimerization and coactivator interactions, which can also be regulated by covalent modifications or protein interactions. The ligand-binding function of nuclear receptors has evolved independently during evolution [60]. Although some orphan nuclear receptors may have lost their ligand-binding properties during evolution, the LBD has an inherent ability to bind ligands and only a small number of mutations are required to change an ordinary transcription factor into a ligand-regulated transcription factor. However, the roles and functions of orphan nuclear receptors and their associated ligands are still being investigated.

1.2. NR4A subfamily of orphan receptors

The human genome contains 48 members of the nuclear receptor superfamily [61]. Besides the classical endocrine receptors and adopted orphan receptors, the nuclear receptor superfamily also includes a larger number of orphan receptors [62]. The NR4A subfamily is a group of orphan receptor with no known endogenous ligands and includes three members: NR4A1, NR4A2, and NR4A3. In 1988, Milbrandt (1988) determined the nucleotide amino acid sequence of NGFR-B while searching for growth factor-regulating protein factors. In the same year, Hazel discovered NR4A1 during studies on growth factor stimulation of 3T3 fibroblast cells [63]. In 1992, Law and coworkers (1992) identified NR4A2 as a novel brain-specific transcription factor. Two years later, Ohkura and coworkers (1994) cloned a novel thyroid/steroid receptor superfamily gene called NR4A3 from cultured rat neurons. All NR4A members can be influenced by both synthetic receptor ligands and substances that do not directly bind the receptor, and they

act through both nuclear and extranuclear pathways [64]. X-ray crystallographic and functional studies showed that bulky hydrophobic amino acid side chains are present in the ligand binding pocket of NR4A2, and this may prevent interactions with an endogenous ligand [65-68]. Members of this subfamily act as ligand-independent nuclear receptors and were initially identified as early response genes induced in response to signals associated with cellular inflammation, proliferation, differentiation and survival [69-71].

1.2.1. Structure and DNA binding

The three members of the NR4A family exhibit high homology and shared structure, including their ligand-independent activation function in the N-terminal region, a DNA-binding domain consisting of two zinc fingers, and a ligand-dependent AF-2 activation domain in the C-terminal region [65]. Their degree of similarity is shown in Figure 4 (rev. in [72]). However, the NR4A subfamily differs from other nuclear receptor families in that they are constitutively active, ligand-independent receptors whose transcriptional activity is determined by the receptor's expression and posttranslational modifications [66, 73]. Initial studies showed that unlike other nuclear receptors, the transcriptional activity of NR4A is independent of the LBD, but instead appears to be dependent on the N-terminal AF-1 domain, which mediates ligand-independent transcriptional activation [66, 74, 75]. Furthermore, the LBD of NR4A members contains hydrophilic surfaces instead of the classic hydrophobic cleft that mediates coactivator recruitment of other nuclear receptors [75]. These early observations suggested that NR4A sub family

members represent a distinct group of transcription factors that do not function in a classic manner.

Figure 1-4. Domain Structure of nuclear receptors and similarities between NR4A1, NR4A2, and NR4A3. Reprinted with permission from [72].

All 3 NR4A members are early immediate-response genes, meaning that they are rapidly induced by various stimuli such as growth factors, inflammation, and cellular stress [71]. Figure 5 illustrates the diverse ways that NR4As activate transcription (rev. in [72]). NR4As interact as monomers with the nerve growth factor-induced protein b (NGF1-b) response element (NBRE) (sequence: AAAGGTCA) in target gene promoters to activate gene transcription [76, 77]. The NR4A homodimer consists of two identical NR4A receptor proteins that bind to specific variants of the Nur response element (NurRE, (AAAT(G/A)(C/T)CA) to activate transcription [78]. In addition, NR4A1 and NR4A2 but not NR4A3 can also form heterodimers with the retinoid X receptor (RXR) to activate transcription in a 9-cis retinoic acid-dependent manner through interaction with the DR-5 element (sequence: AGGTCA-NNNAA-AGGTCA) [79, 80]. Different NR4As can also form heterodimers to synergistically activate transcription [81]. Additionally, NR4A1 interacts with p300, Sp1 or Sp4 transcription factors through interactions with DNA-bound specificity proteins (Sp). As a cofactor for Sp-dependent gene expression, NR4A1 interacts with p300, Sp1 or Sp4 bound to GC-rich promoter sequences in the survivin, β1-, β3-, and β4-integrins, PAX-FOX01, and α5- and α6-integrin genes and

these interactions have been observed in ChIP assay. [82-85]. Previous studies also demonstrated that other nuclear receptors including steroid hormone receptors act as cofactors of Sp1-mediated transactivation [86].

Figure 1-5. Interaction of NR4A with cis-elements, DNA-bound RXR, and DNA-bound-Sp. Reprinted with permission from [72].

In addition to their rapid expression as early-response genes, NR4As are also regulated by posttranslational modifications, which refer to structural protein changes, such as phosphorylation or sumoylation, which can affect its functions and activity. NR4As are phosphorylated at specific serine residues in response to growth factor-dependent activation of various kinases. The kinases that phosphorylate NR4As include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), Jun-N-terminal kinase (JNK), and ribosomal S6 kinase (RSK) [87]. For example, NR4A1 is phosphorylated at serine 350 and serine 354 within the DNA-binding domain, and this inhibits its transactivation activity [88, 89]. This means that site-specific phosphorylation inhibits NR4A1 from activating transcription. Additionally, phosphorylation of NR4A1 at serine 105 results in the export of NR4A1 from the nucleus, providing another mechanism by which phosphorylation can inhibit the transcriptional activity of NR4A1 [90]. Furthermore, all NR4As contain consensus sumoylation sites, which means they can be modified by addition of small ubiquitin-like modifier (SUMO) proteins [91]. Sumoylation of NR4A2 can either induce or inhibit

transcriptional activity, depending on the specific sumoylation site. These posttranslational modifications represent a major mode for controlling gene expression regulated by NR4As.

1.2.2. Functions

1.2.2.1. NR4A-KO mice

Knock out of individual NR4As in mice did not reveal any role for these receptors in causing cancer. While both NR4A1 and NR4A3 are involved in causing apoptosis of B cells and negative selection of T-lymphocytes, NR4A1$^{-/-}$ mice were still viable without any noticeable differences with wild-type mice [92-94]. In contrast, studies with NR4A2$^{-/-}$ mice showed that the receptor is important for the induction of the dopaminergic phenotype, and these mice displayed significant neuronal dysfunction and early mortality [95, 96]. NR4A3$^{-/-}$ mice were produced in two laboratories, and their phenotypes are different. One study showed that the mice died as embryos due to incomplete gastrulation [97], while the other suggested that loss of NR4A3 resulted in inner ear defects [98]. The role of NR4A1 in tumorigenesis was studied in mice by comparing the development of cancer cell implants or xenografts in the presence or absence of NR4A1. For instance, in a syngeneic mouse model that used B16 melanoma cells, the absence of NR4A1 enhanced tumor invasion and metastasis due to an increase in TNF-α secretion and decreased expression of CSF-1R and tumor-infiltrating migratory activity [99]. However, in mice with B16F1 melanoma cells, NR4A1 stimulated tumor growth and enhanced angiogenesis by regulating VEGF expression

[100]. Additionally, NR4A1 expression in mouse MV3 melanoma cells increased the survival and metastasis of circulating tumor cells [101]. Similar results were observed in in vivo and in vitro studies that used LLC and CMT93 colon cancer cells, where the loss of NR4A1 in mice resulted in decreased tumor growth and metastasis [102]. Hence, most studies suggest that NR4A1 exhibits pro-oncogenic activity in solid tumors, except in APC $^{Min/+}$ mice, where NR4A1 loss led to enhanced intestinal tumorigenesis [103]. Double knockout NR4A1$^{-/-}$ and NR4A3$^{-/-}$ mice have a smaller size compared to their wild-type mice and eventually died to acute myeloid leukemia (AML) within a short span of 3 to 4 weeks after birth [104]. In addition, these mice had expanded levels of myeloid progenitor cells and hematopoietic stem cells and decreased expression of the pro-apoptotic proteins c-Jun, Jun-B, Fas-L, and TRAIL. Low or undetectable levels of NR4A1 and NR4A3 were found in leukemia-derived cell lines and leukemic blasts from AML patients, suggesting a potential tumor suppressor-like effect of these genes when combined [104]. In a comprehensive study involving various genotypes, distinct patterns emerged. Wild-type mice (NR4A1$^{+/+}$/NR4A3$^{+/+}$) and single knockout mice (NR4A1$^{+/+}$/NR4A3$^{-/-}$ or NR4A1$^{-/-}$/NR4A3$^{+/+}$) exhibited normal characteristics. However, knockout/heterozygous mice (NR4A1$^{+/-}$/NR4A3$^{-/-}$ or NR4A1$^{-/-}$/NR4A3$^{+/-}$) displayed features consistent with mixed myelodysplastic/myeloproliferative neoplasms (MDS/MPN). These findings were in line with the observations made in double knockout mice, which developed acute myeloid leukemia (AML). Moreover, the MDS/MPN mice showed altered gene expression, including decreased levels of Jun-B,

egr1, and polo-like kinase 2 (Pik2), which were consistent with observations in NR4A1$^{-/-}$ /NR4A3$^{-/-}$ mice [105].

1.2.2.2. Endogenous roles of NR4A

NR4As have important roles in maintaining cellular homeostasis and influencing disease processes. Among these receptors, NR4A1 (Nur77) was initially identified as an inducer of apoptosis in T-cell hybridomas or thymocytes [106, 107]. However, NR4A1 knockout mice do not exhibit defects in T cell receptor-mediated apoptosis, as other apoptosis-inducing factors still function [108]. NR4A1 also regulates adrenocortical function by modulating CYP21 expression. Interestingly, the hypothalamic-pituitary axis functioned normally in NR4A1 knockout mice, suggesting that compensatory factors exist in these mice to compensate for the loss of NR4A1 [109, 110]. In T cells, loss of NR4A1, NR4A2, and NR4A3 impedes Treg cell development leading to multiorgan autoimmune disease, emphasizing the importance of tissue-specific knockout mouse models to further explore the functions of these receptors.

NR4A2 shares high sequence identity and functional similarity with NR4A1, as their DNA-binding domains are more than 92% homologous [111]. Studies have linked NR4A2 to various processes including energy metabolism, atherosclerosis, vascular function, T cell receptor-mediated apoptosis, inflammatory responses, regulation of the hypothalamic-pituitary axis, and reproductive processes [112]. Furthermore, NR4A2 plays a critical role in central nervous system development and homeostasis, with implications for functional working memory and neurological disorders such as

Parkinson's disease [113, 114]. Similar to NR4A1 and NR4A3, NR4A2 regulates target genes by binding cis-elements, including the NGFI-B response element (NBRE) with an AAAGGTCA consensus sequence, and forms monomers, homodimers, or heterodimers with RXR [115, 116]. The transcriptional activity of NR4A2 goes beyond transactivation and includes transcriptional repression through recruitment of nuclear co-repressor proteins, stabilization of histone-DNA binding, and repression of gene expression [117]. The effect of NR4A2 activation on gene expression is highly dependent on cell type and specific signaling events. For example, NR4A2 activation induces apoptosis in cancer cell lines, promotes dopaminergic neuron development and maturation, and suppresses inflammatory responses in macrophages (rev. in [118]). NR4A2 knockout mice exhibit impaired dopamine neuronal differentiation and exhibit various defects similar to NR4A1 knockout mice [95].

NR4A3 also exerts transactivation and interacts with different cis elements, but does not form heterodimers with RXR, which is different from NR4A1 and NR4A2 [79, 119]. While early reports indicated embryonic lethality in NR4A3 knockout mice, subsequent studies have shown that these mice survived but had defects in inner ear semicircular canal and hippocampus development, possibly resulting in neuronal abnormalities and resistance to kainic acid-induced seizures [98, 120].

1.2.2.3. NR4A overexpression and role in multiple diseases

Immune cell exhaustion

NR4As, including NR4A1, NR4A2, and NR4A3, are involved in various inflammatory and immune responses. NR4A1 has been studied extensively in metabolic, cardiovascular, neurological disorders and arthritis, where it generally appears to be protective. Studies have shown that the NR4A receptor plays an important role in T cell development [121] and that NR4A1 specifically regulates genes important for the differentiation of regulatory T cells (Treg cells) [122]. Furthermore, NR4A1 has been found to contribute to the anti-inflammatory effect of apoptotic cells in macrophages [123]. Recent research has indicated that NR4A1 plays a role in regulating the expression of PD-1 and is associated with T cell exhaustion and dysfunction [124]. In mouse models, NR4A1 is highly expressed in T cells exhibiting tolerance or dysfunction. Increased NR4A1 levels hinder the differentiation of effector T cells, while the absence of NR4A1 overcomes T cell tolerance and promotes T cell proliferation, thereby enhancing the effectiveness of antitumor responses. Additionally, NR4A1 deficiency in mice resulted in a decrease in expression levels of PD-1 and TIM-3 in T cells. Mechanistic analysis revealed that NR4A1 preferentially binds to the activator protein 1 (AP-1) transcription factor, leading to the suppression of effector gene expression by interfering with AP-1 function. These findings highlight the significance of NR4A1 in driving T cell dysfunction and suggest that targeting NR4A1 holds promise for augmenting cancer immunotherapy [124].

NR4A2 has constitutive and inducible anti-inflammatory activity in immune cells of the monocyte/macrophage lineage and brain glial cells such as astrocytes and microglia. Its

anti-inflammatory effects target the NFκB signaling pathway, which is involved in the response to inflammatory stimuli [125]. NR4A2 targets inflammatory gene promoters by interfering with NFκB-p65 and recruiting the CoREST co-repressor complex, resulting in transcriptional repression and clearance of NFκB-p65 [125]. These findings suggest that NR4A2 prevents neuronal loss in neurodegenerative diseases such as Parkinson's disease by limiting the production of neurotoxic mediators by microglia and astrocytes [125]. However, NR4A2 can also exert pro-inflammatory effects in synoviocytes associated with arthritis [126].

NR4A2 is also involved in the maturation and differentiation of Th17 T cells, which has important implications for addressing autoimmunity and infection [127]. NR4A2 is upregulated in rheumatoid arthritis, induced by various inflammatory mediators, and acts as a trans-repressor of the NFκB pathway, thereby limiting the inflammatory response [128]. Regulation of the forkhead transcription factor Foxp3 by NR4A2 is required for Treg cell differentiation and occurs through direct interaction with Runx1 [129]. Induction of NR4A2, as well as other anti-inflammatory mediators, is important for attenuating responses to inflammatory inducers [114]. Overall, NR4A2 appears to have broad effects on regulating inflammation and addressing inflammatory signaling in immune cells and glial cells.

NR4A3, like other NR4A receptors, is induced by stress and upregulated under inflammatory conditions. It also plays a crucial role in T cell receptor-induced apoptosis.

Mouse knockout studies have shown that NR4A1 and NR4A3 are key factors in regulating Treg cell homeostasis and preventing autoimmunity [121]. Mice lacking NR4A1 and NR4A3 showed reduced Treg cell development and died within weeks, highlighting the importance of these receptors in Treg cell function and immune regulation [121].

Generally, NR4A1 tends to be protective under inflammatory conditions, NR4A2 exhibits anti-inflammatory activity but also causes inflammation in certain conditions, and NR4A3 plays a key role in T cell regulation and apoptosis. Understanding the function and interactions of NR4A receptors during inflammation may lead to the development of targeted therapies for various inflammatory diseases and conditions.

Metabolic disease

NR4A1, NR4A2, and NR4A3 are highly expressed in obese individuals and decreased after weight loss. These receptors are induced by cAMP and glucagon, and overexpression of NR4A1 leads to increased expression of gluconeogenesis genes [130]. In mouse models, NR4A1 activation increases blood and liver glucose levels, whereas inhibition of NR4A1 decreases glucose levels [130]. However, NR4A1 knockout mice fed a high-fat diet exhibited increased insulin resistance and hepatic steatosis, suggesting a complex role for NR4A1 in metabolic disease [131]. In diabetic mice, NR4A1 expression correlated with higher blood glucose levels. Researchers have identified ligands that bind NR4A1, such as cytosporone B (CsnB) and ethyl [2,3,4-trimethoxy-6-

(i-octanoyl)phenyl]acetate (TMPA), that have differential effects on glucose levels. TMPA decreased blood glucose levels and inhibited hepatic gluconeogenesis in diabetic mice, whereas CsnB increased glucose levels and induced gluconeogenesis [132].

NR4A2 plays a crucial role in regulating expression of multiple genes related to metabolism and gluconeogenesis, suggesting its importance in metabolic diseases. Its hepatic expression is induced by a variety of compounds, including cAMP, glucagon, fatty acids, glucose, insulin, cholesterol, and thiazolidinediones [129]. NR4A2, along with NR4A1 and NR4A3, is upregulated in the liver during dietary restriction, highlighting the role of a subset of NR4As in actively regulating metabolism in response to dietary inputs [133]. Furthermore, increased expression of specific genes related to glucose utilization and insulin sensitivity was observed in muscle following dietary restriction and this was associated with enhanced activity of NR4A1 and NR4A3. NR4A2 also regulates other metabolism-related genes, including AbcG5/8, ApoB/E, Fas, Fbp1/2, Glut4, Ucp2/3, and Pgc1a [129].

When NR4A3 is inhibited in C2C12 skeletal muscle cells, it results in changes in gene expression, indicating a shift in gene activity from oxidative to anaerobic [134]. In contrast, studies in NR4A3-overexpressing mice showed that NR4A3 promotes the development of type II muscle fibers and enhances fatigue resistance [135]. Additionally, NR4A3 has been implicated in determining the difference between high and low running abilities in rodents [136]. NR4A3 stimulates cAMP production in

hepatocytes and in the liver of fasted mice [130] and NR4A3 levels are elevated in obese patients [137]. However, the specific role of NR4A3 in mouse models of obesity and type 2 diabetes (T2DM) has not been extensively studied. Generally, the NR4A family has great potential as targets for the development of therapeutic drugs to address metabolic diseases and their associated complications.

Cardiovascular disease

NR4A members are affected in cardiovascular disease due to their association with chronic inflammation. Among the NR4A subfamily, NR4A3 has been proposed to promote the development of atherosclerotic lesions, whereas NR4A1 and NR4A2 attenuate atherosclerosis [116]. NR4A1 is expressed and functional in various cell types involved in arterial vascular injury, including vascular smooth muscle cells, endothelial cells, macrophages, and monocytes (rev. in [118]) Studies have shown that NR4A1 is induced in smooth muscle cells treated with growth factors and cytokines, as well as in mouse models of atherosclerotic lesions [138]. Perturbation of smooth muscle cells increases expression of NR4A1, and experimental knockdown or overexpression of NR4A1 suggests its inhibitory role in cell proliferation [139]. The antioxidant α-lipoic acid has been shown to inhibit carotid intimal hyperplasia in rats by inducing cytoplasmic NR4A1, and the protective effect was attenuated when NR4A1 was knocked down [140]. NR4A1 also plays a role in α-lipoic acid-induced apoptosis in vascular smooth muscle cells and in reactive oxygen species-induced apoptosis in neonatal heart cells cultured under conditions similar to a high-fat diet [141]. In

endothelial cells, NR4A1 is induced by multiple factors and contributes to cell proliferation and angina pectoris [142]. Furthermore, NR4A1 is expressed in macrophages in areas of plaque formation, and its increased expression or activation has been associated with reduced foam cell formation and atherosclerotic plaque development in cellular and mouse models [143]. On the other hand, loss of NR4A1 enhances atherosclerosis, Toll-like receptor signaling, and pro-inflammatory macrophages [144]. NR4A1 is also involved in inflammatory and repair responses during healing after myocardial infarction. The protective role of NR4A1 in cardiovascular disease differs from its disease-promoting role in metabolic disease.

NR4A2 exhibits anti-mitogenic effects in smooth muscle cells, thereby combating atherosclerotic plaque formation [145]. Furthermore, NR4A2 suppresses the expression of inflammatory genes in macrophages by inhibiting NFκB signaling, contributing to their anti-atherosclerotic activity [125]. Macrophages play a crucial role in atherosclerosis by releasing cytokines and growth factors that exacerbate local inflammation and activate smooth muscle cells, leading to excessive lipid uptake and lipid-rich foam within plaques Cell formation [146]. NR4A2 reduces the uptake of oxidized LDL by macrophages and reduce the expression of pro-inflammatory cytokines and chemokines, further supporting its protective effect against cardiovascular disease [147].

NR4A3 is also expressed in atherosclerotic lesions and induced by various stressors in smooth muscle cells (rev. in [118]). Knockdown experiments showed that NR4A3 promotes proliferation of these cells by regulating genes involved in cell proliferation [148]. Compared with NR4A1, NR4A3 enhanced neointimal hyperplasia, the thickening of the innermost layer of arteries. NR4A3 also inhibits NFκB signaling in vascular smooth muscle cells, suggesting an anti-inflammatory function in this cell type [149]. In endothelial cells, NR4A3 is induced by VEGF and plays a role in cell proliferation. NR4A3 regulates the expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells and is involved in monocyte adhesion [150]. In in vivo models of atherosclerosis, NR4A3 regulates srecruitment of monocytes to vessel walls and promotes macrophage recruitment. In contrast, NR4A1 showed opposite responses, suggesting that these two NR4A receptors play different roles in the atherosclerosis [143].

These findings highlight the complex involvement of NR4As in atherosclerosis, with NR4A1 and NR4A2 conferring protection against the disease and NR4A3 contributing to its progression. Understanding the exact mechanisms and interactions of NR4As in atherosclerosis may provide potential therapeutic targets for cardiovascular diseases.

Other inflammatory diseases -uterine fibroids

Uterine fibroids (UF) are benign tumors of the uterine myometrium. The estrogen and progesterone receptors are primarily responsible for the maintenance and progression of

UF, and their expression is up-regulated when activated by estrogen and progesterone [151]. However, mRNA expression profiling studies have shown that NR4A members are drastically down-regulated in UF, contributing to the proliferative and profibrotic effects observed in UF [151]. This suggests that the expression status of NR4A may serve as a molecular marker in the onset and progression of UF. Furthermore, functional genomic and proteomic analysis has shown that many genes contributing to UF, such as TGF-β3, SMAD3, and collagen genes, are negatively regulated by NR4A members. Overexpression of NR4A receptors in primary UF cells decreased the expression of these genes, suggesting a potential therapeutic approach for UF treatment.

1.2.3. NR4A ligands

1.2.3.1. NR4A1 ligands

Cytosporone B and analogs

The first identified NR4A1 ligand was cytosporone B (CsnB). It is a metabolite from octaketofungi and was first identified as a ligand for NR4A1 by Wu and colleagues (2008) when they screened a natural product library (Figure 6). As reviewed by Safe and coworkers (2021), CsnB induces NR4A1-dependent transactivation in human gastric BGC-823 cells and directly binds the ligand-binding domain (LBD) of NR4A1 with a KD of 7.4 × 10−7 M. Results of CsnB-induced transactivation in BGC-823 cells suggest a nuclear function for NR4A1; however, CsnB also induces nuclear export of NR4A1 in approximately 70% of cells, forms a pro-apoptotic bcl2-NR4A1 complex and induces cell death and inhibits tumor growth in vivo [152]. At a dose of 50 mg/kg, CsnB acts as

an NR4A1 agonist and enhances blood glucose levels and hepatic expression of gluconeogenesis genes and NR4A1 in a mouse model [152]. Subsequent studies identified synthetic analogs of CsnB, which are also NR4A1 ligands, and these compounds enhanced NR4A1 expression, activated nuclear NR4A1 as well as nuclear export of the receptor [153]. The crystal structure of the NR4A1 (LBD)–CsnB complex shows that the ligand bridges the LBD of the NR4A1 homodimer. Essential amino acids for this novel interaction include residues Asp481, GIn571, and Arg572 [154]. These results were obtained in a study on the role of NR4A1 homodimers as inhibitors of breast cancer progression by inhibiting genes involved in the uptake of fatty acids by cancer cells. Loss of NR4A1 in genetic and carcinogen-induced mouse models of breast cancer resulted in enhanced tumorigenesis [154], in contrast to other studies showing a pro-oncogenic effect of NR4A1 in certain breast cancer cell lines [155, 156]. CsnB has been used extensively to study the role of NR4A1 in a variety of inflammatory diseases in mouse models in the presence or absence of ligands.

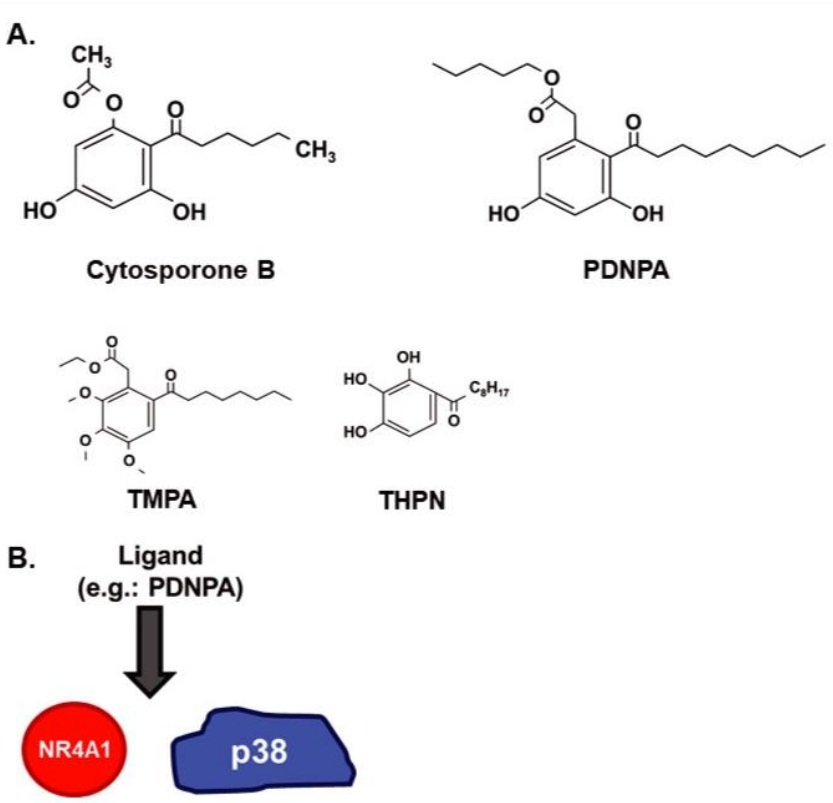


Figure 1-6. Structures and mechanisms of action of Cytosporone B and analogs. (A) Structures of CsnB and related compounds. (B) Mechanisms associated with PDNPA-inducing dissociation of NR4A1 from p38. Reprinted with permission from [157].

n-Pentyl 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl acetate (PDNPA) is another compound that binds to the ligand-binding domain (LBD) of NR4A1. PDNPA has a similar structure to CsnB. Studies have shown that PDNPA inhibits the interaction between NR4A1 and p38 and then decreases inflammation induced by lipopolysaccharides (LPS). The binding of PDNPA to NR4A1 LBD involves specific amino acids such as Leu437, Ser441, and Asp549. However, it is important to note that PDNPA does not interact with the canonical binding pocket of NR4A1, meaning that its effects are specific for the inhibition of NR4A1-p38 interactions (rev. in [157]). Interestingly, PDNPA also binds NR4A2 and NR4A3, but this binding does not result in phenotypic effects. Overall,

PDNPA is a selective inhibitor of NR4A1-p38 binding and has the potential to be used as an anti-inflammatory agent.

Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl acetate (TMPA) is a compound that inhibits the interaction between NR4A1 and LKB1, which results in the phosphorylation (activation) of AMPK and inhibition of gluconeogenesis [158]. Gluconeogenesis is a process in which glucose is synthesized from non-carbohydrate sources such as amino acids and lipids. By inhibiting this process, TMPA can decrease blood glucose levels in mouse models of insulin resistance [152]. X-ray crystallographic analysis of TMPA–NR4A1 (LBD) interactions shows that TMPA binds close to the surface of the NR4A1 (LBD) and not deep within the binding pocket. The ligand primarily interacts with side chains of several amino acids (Arg515, Glu445, Thr595, His372, Arg450, Tyr453, Leu492, and Val498). Mutational analysis confirmed that Thr595 is necessary for NR4A1–LKB1 interactions, whereas Cys566 is required for TMPA binding to NR4A1.The functional differences between PDNPA and TMPA may be due to their differences in binding to NR4A1. PDNPA competes with p38 for binding to the LBD of NR4A1 and inhibits NR4A1-p38 interaction, whereas TMPA inhibits the interaction between NR4A1 and LKB1. Hence, TMPA acts as an antagonist, but PDNPA is an agonist in this process (rev. in [157]).

Another CsnB analog, known as 1-(3,4,5-trihydroxyphenyl)nonan-1-one (THPN), was also identified by Wu and colleagues (2014). THPN binds to NR4A1 and triggers

autophagic cell death by activating mitochondria in certain melanoma cell lines. Unlike CsnB, THPN does not induce the nuclear export of NR4A1. Instead, it targets cytosolic NR4A1 found in melanoma cells to mitochondria. THPN interacts with surface residues, Arg563 and Ser553, around the binding cavity of NR4A1. These sites are required for THPN-bound NR4A1 to interact with the mitochondrial protein, Nix, which triggers autophagy. Although THPN does not directly bind to Nix, its interaction with NR4A1 is necessary for NR4A1-Nix binding and the activation of autophagic cell death through specific mitochondrial interactions. CsnB and its analogs exhibit structure-dependent interactions with different amino acids in the NR4A1 binding AF2 domain. This variability may contribute to their diverse agonist and antagonist activities, as well as their interactions with other NR4A1 interactants such as binding proteins.

CDIMS

Bis-Indole derivatives (CDIM) are a series of synthetic compounds developed in the Safe laboratory and these potent NR4A1 ligands act as an NR4A1 antagonist in cancer cells. The structure of CDIM and multiple pro-oncogenic pathways/genes regulated by NR4A1 in solid tumors that are inhibited by CDIM/NR4A1 antagonists are illustrated in Figure 7. NR4A1 is overexpressed in many solid tumors, and in breast, colon, lung, and ovarian tumors. NR4A1 is a negative prognostic factor for patient survival or recurrence (rev. in [159]). Knockdown of NR4A1 in most solid tumor-derived cell lines results in decreased growth, survival, migration, invasion, and associated genes, demonstrating that NR4A1 is a pro-oncogenic factor. In alveolar rhabdomyosarcoma (ARMS), NR4A1

regulates multiple pro-oncogenic pathways/genes including the PAX3-FOX01 fusion gene, which is the major oncogenic transcriptional driver of this tumor [160]. Bis-indole-derived (CDIM) compounds were initially identified as PPARγ ligands, and subsequent structure-activity studies showed that some CDIMs bound to NR4A1 and inhibited NR4A1-dependent transactivation in multiple cancer cell lines (rev. in [159]). NR4A1-active CDIMs inhibited most of the pro-oncogenic pathways and associated genes in colon, pancreatic, lung, breast, rhabdomyosarcoma, kidney, and endometrial cancer cell lines (rev. in [157]). Therefore, CDIMs were classified as NR4A1 inverse agonists based on their downregulation of the functional pro-oncogenic responses and genes in solid tumors. Initial studies focused on 1,1-bis(3′ -indolyl)-1-(p-hydroxyphenyl)methane (CDIM8), which inhibits cancer cell and tumor growth in athymic nude mouse xenograft models. However, CDIM8 is rapidly metabolized, and blood levels are low [161]. Recent studies showed that adding substituents ortho to the 4-hydroxyl at C-3 and C-5 in the phenyl ring resulted in a buttressing effect, decreasing metabolic conjugation of the hydroxyl group, and enhancing potency of tumor growth inhibition in athymic nude mouse xenograft models [162]. Several oxidized analogs of CDIMs have also been reported, and these compounds are potent inhibitors of cancer cell growth and appear to be more active than their parent precursors in several cancer cell lines and in vivo Fields [163, 164]. The oxidized mesylate derivative of DIM-C-pPhCF3 bound NR4A1, and key interactions with side chains of His372 and Tyr453 located in helices 1 and 5, respectively, were major binding determinants.

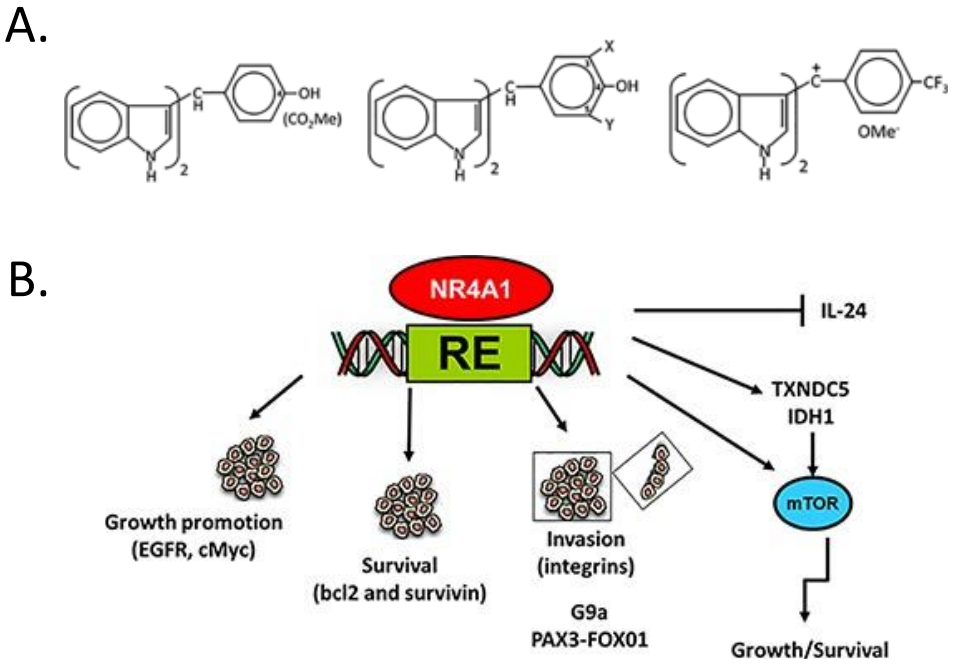


Figure 1-7. Structure of NR4A1 ligands. Structure of bis-indole-derived compounds (CDIM) NR4A ligands (A) and multiple pro-oncogenic pathways/genes regulated by NR4A1 in solid tumors that are inhibited by CDIM/NR4A1 antagonists (B). Reprinted with permission from [157].

The modulation of gene expression by CDIMs involves multiple pathways and genes. And is dependent on formation of liganded NR4A1 monomers and dimers that bind to cognate NBRE and NuRE sequences or Sp1/Sp4 bound to a GC-rich promotor. Additionally, another mechanism of NR4A1-dependent transactivation occurs through NR4A1-RXR complexes that interact with a DRE motif. Recent research has also reported that NR4A2 interacts with novel promoter sequences and may also bind NR4A1 [165]. Genomic analysis of NR4A1-regulated genes reveals that many of these genes, such as survivin and epidermal growth factor receptor (EGFR), are Sp1-regulated genes. Knockdown of NR4A1 or treatment with CDIM/NR4A1 antagonists decreases expression of survivin in pancreatic cancer cells. Further analysis shows that levels of

survivin depend not only on Sp1 but also on NR4A1 and p300. Knockdown of Sp1, NR4A1, or p300 decreases survivin expression, indicating that NR4A1 acts as a nuclear cofactor [86]. Subsequent studies demonstrate that NR4A1/Sp1 and/or NR4A1/Sp4 regulate multiple genes through their NR4A1/Sp interactions with GC-rich promoters. These include PAX3-FOX01, PD-L1, G9a, and several integrins [82, 84, 85, 166, 167]. TGFβ plays a crucial role in invasion of breast and lung cancer cells and this involves phosphorylation and subsequent nuclear export of NR4A1, which interacts with a proteasome complex that degrades inhibitory SMAD7 [64, 168]. CDIMs interact with NR4A1 to inhibit nuclear export, which is accompanied by decreased degradation of SMAD-7 and inhibition of TGFβ-induced invasion [169, 170]. CDIM NR4A1 antagonists enhance apoptosis in RD embryonal rhabdomyosarcoma (ERMS) cells by interacting with constitutive cytosolic NR4A1, resulting in apoptosis. The oxidized CDIM+ compounds induce nuclear export of NR4A1, which forms a pro-apoptotic NR4A1-bcl2 complex as described for celastrol. This pathway is also activated by other pro-apoptotic agents that do not directly bind NR4A1 [141].

Celastrol

Celastrol is a natural compound that belongs to the triterpenoid family and has been found to have anti-cancer properties. Celastrol binds NR4A1 with a KD value of 0.29 μM and inhibits NR4A1-dependent transactivation [171, 172]. Molecular modeling studies have revealed that celastrol interacts with a surface region near the LBD of NR4A1 [158], similar to TMPA. Additionally, like TMPA, celastrol inhibits high-fat

diet-induced chronic inflammation and weight gain. Celastrol triggers anti-inflammatory responses by inducing nuclear export of NR4A1, where it interacts with mitochondrial TRAF2 [171]. The modeling of celastrol-NR4A1 binding has shown that the compound interacts with GIn547 and Asp499, and the KD value is 0.32 μM [158].

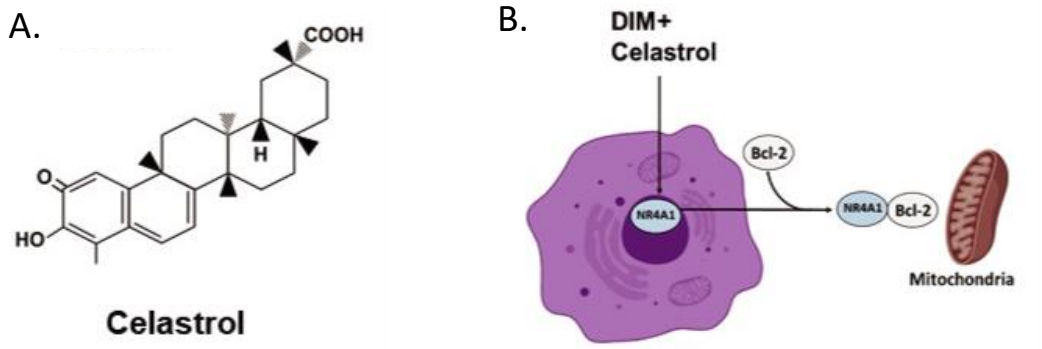


Figure 1-8. Structures and mechanisms of action of celastrol. (A) Structures of celastrol. (B) Effects of celastrol and oxidized DIM compounds inducing nuclear export of NR4A1 which forms a pro-apoptotic NR4A1–bcl2 complex that target mitochondria. Reprinted with permission from [141].

Others NR4A1 ligands

Arachidonic and docosahexaenoic acids are unsaturated fatty acids that also bind NR4A1 [173]. The unsaturated fatty acids play a role in stabilizing NR4A1 oligomer complex formation. Prostaglandin A2 is another endogenous compound that binds NR4A1 and forms a covalent adduct at Cys566 [174]. This compound induces NR4A1-dependent transactivation in human bronchial epithelial cells and also binds NR4A3, making it a dual receptor ligand [175]. A computational modeling study identified 2-imino-6-methoxy-2H-chromene-3-carbothioamide (IMCA) as an NR4A1 ligand that induces nuclear export of NR4A1 in medullary thyroid cancer cells [176]. Additionally, some flavonoid compounds such as kaempferol and quercetin also bind NR4A1 and

show anticancer activity. These flavonoids bind NR4A1 and inhibit NR4A1-dependent transactivation by decreasing PAX3-FOXO1-G9a pathway and mTOR signaling to suppress RMS cell growth [177].

1.2.3.2. NR4A2 ligands

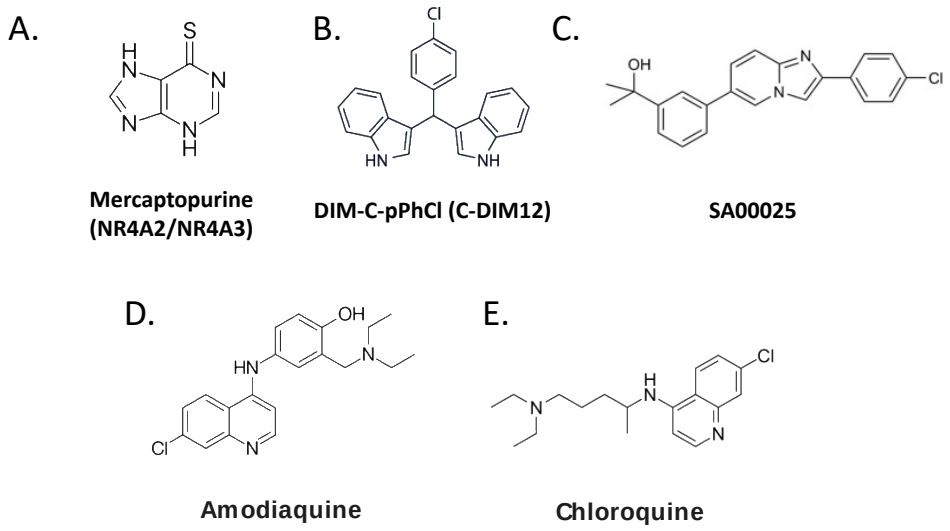


Figure 1-9. Examples of ligands that bind NR4A2. A. Mercaptopurine is identified as both NR4A2 and NR4A3 ligand. B. DIM-C-pPhCl (C-DIM12). C. SA00025. D. Amodiaquine. E. Chloroquine. Reprinted with permission from [118, 178].

Although NR4A2 is considered as an orphan nuclear receptor whose activation does not require ligand binding [179]. Figure 9 illustrates several different classes of compounds that have recently been reported to act as Nurr1 agonists. Due to the unique function of NR4A2 in neuroinflammation and neuronal cell death, they have the potential therapeutic protective effects on several CNS disorders [180].

Mercaptopurine (6-mercaptopurine or 6-MP) is a drug used to treat leukemia and was identified as the first NR4A2/NR4A3 ligand. This compound directly binds the N-terminal AF-1 domain, thereby stimulating NR4A2/NR4A3 [181, 182]. Studies have shown that 6-MP exhibits promising results for reducing cerebral infarction in a rodent model of permanent middle cerebral artery occlusion (pMCAO). In addition, it disrupts production of IL-1β and TNF-α in cerebrospinal fluid (CSF) and serum [183]. A recent study demonstrated an anti-inflammatory role of 6-MP in BV-2 microglia-induced inflammation after exposure to lipopolysaccharide (LPS). This component significantly reduces TNF-α production and inhibits the transactivation activity of NF-κB and TNF-α promoters by preventing p65 phosphorylation at Ser276 and p65 acetylation at Lys310. Chromatin immunoprecipitation analysis revealed that 6-MP reduced LPS-induced histone H3 acetylation in chromatin regions surrounding the TNF-α promoter, ultimately leading to reduced p65/coactivator-mediated TNF-α gene transcription. In addition, 6-MP enhances expression of NR4A1. Knockdown of NR4A1 expression in cells treated with 6-MP resulted in significantly reduced inhibitory effects of the drug on TNF-α production. Furthermore, 6-MP delays TNF-α mRNA translation by preventing the LPS-activated PI3K/Akt/mTOR signaling cascade [184].

Numerous studies have shown that some 1,1-Bis(3-indolyl)-1-(substitutedphenyl) methane (C-DIM) analogs can regulate the expression of NR4A subfamily in various cancer cells [185-187]. 1,1-bis(3′-indolyl)-1-(p- chlorophenyl)methane (DIM12) exhibits a higher affinity for NR4A2 compared to other C-DIM analogs. Modeling

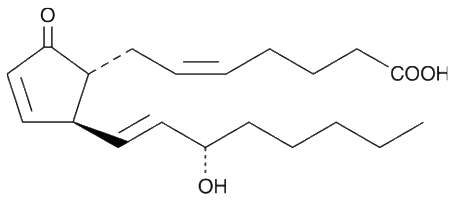
studies suggest that DIM12 binds to the coactivator region of the NR4A2 [188]. In pancreatic cancer cells, transactivation studies have shown that DIM12 activates multiple genes and NR4A2-responsive constructs [189, 190]. However, DIM12 also exhibits functional antagonist activity and potently inhibits cancer cell growth and survival [191, 192]. In colon cancer, prostaglandin E2 (PGE2), a product of cyclooxygenase 2 (COX-2), acts oncogenically and induces NR4A2 expression in both in vivo and in vitro models. This induction of NR4A2 results in changes in gene expression, including increased expression of osteopontin and fatty acid oxidation (FAO) (rev. in [159]). Activation of NR4A2 by PGE2 in stromal cells enhances NR4A2/RXR-mediated prolactin expression. The resulting tumor stromal prolactin signaling pathway plays a role in the initiation of prostate cancer and can be blocked by COX-2 inhibitors [193]. PGE2 enhances expression of NR4A2 regulatory proteins, including tyrosine hydroxylase and dopamine transporter. In addition, DIM12 exhibits NR4A2-mediated CoREST recruitment and suppresses NF-κB-mediated inflammatory gene expression in the substantia nigra (SN) BV-2 cells [194, 195]. Several studies have shown that DIM12 inhibits MPTP-induced glial activation and neuronal loss in mice [196]. In vitro assays confirmed the efficacy of DIM12 as an activator of NR4A2, and computational modeling indicated a high affinity between DIM12 and the three-dimensional structure of human NR4A2.

SA00025 is a recently discovered compound that acts as a novel NR4A2 agonist with an EC50 of 2.5 nM in PD models induced by inflammatory stimulant poly (I:C) and 6-

hydroxydopamine (6-OHDA). Furthermore, SA00025 regulates expression of several dopamine target genes, including tyrosine hydroxylase, vesicular monoamine transporter, dopamine transporter, aromatic L-amino acid decarboxylase, and c-Ret in SN [197].

The 4-amino-7-chloroquine derivatives such as amodiaquine and chloroquine have also been identified as NR4A2 agonists [198]. These compounds directly interact with the NR4A2-LBD through physical binding as determined using a Biacore S51 SPR sensor, fluorescence quenching analysis, a radioligand-binding assay using [3H]-CQ, and nuclear magnetic resonance assays. The effective concentrations (EC50) of AQ/CQ for NR4A2 activation range from 20 to 50 μM [198]. Amodiaquine suppressed LPS-induced inflammatory gene expression in BV-2 cells and prevented microglial activation in the substantia nigra and improved nervous system function in 6-OHDA hemiparkinsonian mice. In addition, daily intraperitoneal injections of amodiaquine after induction of intracerebral hemorrhage in mice inhibits the activation of perihematoma microglia, inhibits the upregulation of multiple inflammatory factors such as IL-1β, CCL2, and CXCL2, and attenuates neurological deficits [125].

1.2.3.3. NR4A3 ligands



Prostaglandin A2

Figure 1-10. Prostaglandin A2 (PGA2) as an NR4A3 ligand.

Kagaya and colleagues (2005) showed that Prostaglandin A2 (PGA2) activates NR4A3-dependent transcription and binds to NR4A3 in the nucleus. They observed that PGA2 activates NR4A3-dependent transcription even in the absence of LBD, suggesting that PGA2 may act as a transactivator rather than a specific ligand for the receptor. In addition, PGA2 has minimal effects on NR4A3-dependent transcription compared to other ligands for NR4As. NR4A3 is involved in a variety of cellular processes, including leukocyte apoptosis, neural differentiation, and cell growth. PGA derivatives have also been implicated in cell cycle regulation, apoptosis and growth inhibition. Studies have demonstrated that PGA2 induces p21Cip1 expression, represses cyclin D1 expression, and induces cell death, especially in splenocytes derived from NR4A3-overexpressing transgenic mice, suggesting that NR4A3 expression levels influence the effects of PGA2.

1.2.4. NR4A and cancer

1.2.4.1. Blood-derived cancer

As discussed above the combined NR4A1$^{-/-}$ and NR4A3$^{-/-}$ double knockout mice die from acute myeloid leukemia (AML) in 3 to 4 weeks, NR4A1 and NR4A3 in combination exhibit tumor suppressor-like activity in blood-derived cancers [104]. In a lymphoma study, NR4A1 and NR4A3 expression was lower in patients with follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) compared to their cells of origin. Reduced NR4A1 levels are associated with aggressive FL and DLBCL and poorer overall patient survival. NR4A2 expression was only detected in some samples and showed similar levels in tumor and non-tumor tissues [199]. However, NR4A3 was overexpressed in DLBCL patients who responded well to chemotherapy, whereas overexpression was not observed in non-responders [200]. Lower expression of NR4A1/NR4A3 correlated with decreased expression of apoptotic genes such as TRAIL, Puma, and Bim. Transfection of NR4A1 expression plasmids into SuDHL4 lymphoma cells resulted in a marked increase in apoptosis and induction of pro-apoptotic genes. Notably, in the germinal center B-cell-like subtype of DLBCL, high cytoplasmic NR4A1 levels were associated with increased patient survival and this was linked to the ERK1/2 pathway [201]. Another study showed that low NR4A3 expression in aggressive lymphomas was also associated with poor patient survival. Overexpression of NR4A3 in lymphoma cell lines induced apoptosis, highlighting the tumor suppressor role of NR4A1 and NR4A3 through regulation of pro-apoptotic genes. In pediatric pre-B acute lymphoblastic leukemia (Pre-B-ALL) patients, NR4A3 expression was associated with

improved overall and event-free survival [202]. However, the expression of NR4A1 in mantle cell lymphoma (MCL) exhibits different characteristics. NR4A1 is predominantly localized in the nucleus and exhibits higher levels compared to normal B cells [203]. A strong correlation was found between the expression of NR4A1 and Bruton's tyrosine kinase, a key factor in the oncogenic properties of MCL. Knockdown of NR4A1 in MCL cells enhances drug-induced cell killing, and genomic analysis supports its tumor-promoting activity.

Loss of NR4A1 and NR4A3 is implicated in the development and progression of leukemias and lymphomas, except for mantle cell lymphoma (MCL) [204]. Histone deacetylase (HDAC) inhibitors induce NR4A1 and NR4A3 expression in leukemia cells and this results in activation of proapoptotic pathways and enhanced histone acetylation [205]. In vivo studies support the gene dosage effect of NR4A1 [105]. Genome-wide mapping revealed that NR4A1 targets 685 genes in AML cells and cooperates with distal ETS enhancers such as ERG and FLI-1 to regulate the transcription [206]. Chemical screening identified dihydroergotamine (DHE) as a drug that induces NR4A expression and inhibits AML cell growth. Mechanistic studies demonstrated that DHE reverses promoter-paused RNA polymerase II, recruits the super elongation complex, and increases gene expression [207]. Fenretinimide induced NR4A1 expression and nuclear export, resulting in apoptosis through interaction with Bcl-2 [208]. Thapsigargin induced NR4A3 levels in lymphoma cells, mimicking the effects of NR4A3 overexpression and this resulted in the inhibition of cell growth and induction of apoptosis [209]. CsnB was

identified as an NR4A1 ligand and induced apoptosis in lymphoma and immortalized B cells. However, in MCL cells, NR4A1 exhibited pro-oncogenic activity, and treatment with 1,1-bis(30-indolyl)-1-(4-hydroxyphenyl)methane (DIM-C-pPhOH, DIM8), an NR4A1 antagonist, inhibited growth and enhanced ibrutinib-induced cytotoxicity [210]. Overall, NR4A1 and NR4A3 display tumor-suppressive activities in leukemias and certain lymphomas, and drug-induced expression of these receptors holds potential as a treatment strategy. However, MCL stands as an exception due to the tumor promoter-like activity of NR4A1, and the underlying reason for these cell type differences is still unclear.

1.2.4.2. Solid tumors

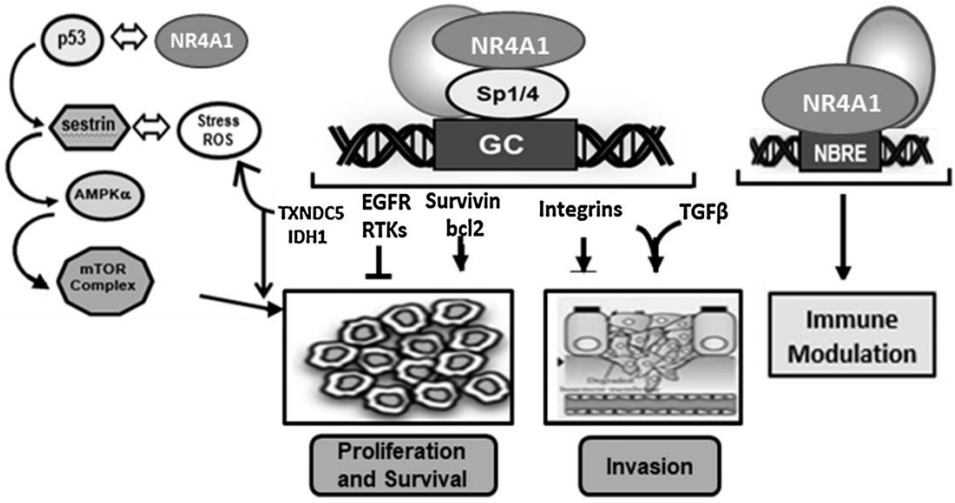


Figure 1-11. NR4A-regulated pathways/genes expression in solid tumors. These results were derived primarily from knockdown studies (of NR4A1) and were observed in multiple cancer cell lines. Reprinted with permission from [72].

The expression, prognostic value, function, compound/ligand effects, and mechanism of action of NR4A have been extensively studied in solid tumors, with most studies focusing on NR4A1. There is evidence that NR4A1 is overexpressed in several solid tumor types, including breast, lung, pancreatic, ovarian, colon, endometrial, cervical, and gastric cancers, rhabdomyosarcoma, and melanoma [72, 82, 84]. High expression of NR4A1 in some of these cancers predicts poor patient survival or prognosis (rev. in [72]) Most studies have shown that NR4A1 is tumor-promoting in solid tumor-derived cell lines, indicating a significant difference between the tumor-suppressor-like activity of NR4A1 in hematologically-derived cancers [211]. NR4A1 plays a role in regulating cell proliferation, survival, migration/invasion and epithelial-mesenchymal transition in breast, colon, pancreatic, renal, lung, rhabdomyosarcoma, melanoma and endometrial cancer cells effect (rev. in [72]). Studies in the Safe laboratory have demonstrated that NR4A1-regulated genes, thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1), are crucial for maintaining high mTOR signaling and reducing intracellular reactive oxygen species (ROS) and ER stress [82, 160, 210]. Knockdown of NR4A1 by RNA interference reduces expression of TXNDC5 and IDH1, leading to the induction of ROS, ER stress, and inhibition of mTOR signaling through sestrin 2-mediated activation of AMPK and inhibition of mTOR. This has also been observed in various cancer cell types, including pancreatic, breast, lung and renal cancer and rhabdomyosarcoma (rev. in [159]), as well as in endometriotic cells [212]. NR4A1 has a tumor-specific role in alveolar rhabdomyosarcoma (ARMS), where it regulates the PAX3-FOX01 fusion oncogene critical for ARMS cell growth [213]. NR4A1 acts as a

nuclear transcription factor or coactivator to regulate target gene expression, and knockout and overexpression studies have provided substantial evidence that NR4A1 regulates solid tumor-derived cancer cell proliferation, survival, and migration/invasion (Figure 11) (rev. in [159]).

NR4A1 has been shown to bind to bcl-2 through the loop region between the BH4 and BH3 domains of the bcl-2 [214], and a site adjacent to the BH3 peptide-binding cleft is also involved in NR4A1-bcl-2 interaction [215].Short NR4A1 peptides and paclitaxel that interact with bcl-2 mimic the proapoptotic effects of NR4A1, indicating that small molecules targeting the NR4A1-bcl-2 interaction site could be a novel class of apoptosis inducers [216, 217]. Insulin-like growth factor-binding protein 3 (IGFBP3) directly interacts with NR4A1, leading to nuclear export and mitochondrial targeting of the receptor, which is associated with activation of Jun N-terminal kinase (JNK) and inhibition of Akt [218, 219]. In melanoma cells, NR4A1 protects the mitochondrial functional protein subunit TPβ from oxidation, enhancing the survival of fatty acid oxidation in melanoma cells maintained in low glucose medium [101]. The novel NR4A1-binding compound 1-(3,4,5-trihydroxyphenyl)nonan-1-one (THPN) induces nuclear export of NR4A1 to the mitochondria of melanoma cells, where it interacts with Tom40 and Tom70, enhancing turnover of VDAC1 that disrupts pore complexing substance. NR4A2 is also an oncogene in solid tumor-derived cell lines and plays a role in cancer cell proliferation, survival and migration/invasion (rev. in [72]). NR4A3 exhibits tumor suppressor-like activity in most blood-borne cancers but tumor-promoting

activity in acinar cell carcinoma (a salivary gland tumor), where it is associated with increased cell proliferation and regulation of genes that cooperate with the MYB oncogene [220, 221]. Despite their structural similarities, NR4A members display paradoxical cellular context-dependent activities and are differentially affected by drugs and receptor ligands and this is partly due to their interactions with other proteins in the cell and intracellular trafficking. Kurakula and colleagues (2014) found that NR4A1, NR4A2 and NR4A3 interact with unique sets of proteins that contribute to their distinct roles in different cell and tissue types. Another key distinguishing feature of NR4A members is their ability to function both inside and outside the nucleus. After activation by various reagents, ligands or stimuli, NR4As can translocate from the nucleus to the cytoplasm and even to the plasma membrane, where they interact with other proteins to regulate cellular processes. For example, studies have shown that the action of certain apoptosis inducers is due to the nuclear export of NR4A1, whereas this is not the case for NR4A2 or NR4A3 [222]. Generally, the complex interactions between NR4As and other proteins and their ability to function both inside and outside the nucleus contribute to their diverse and cell context-dependent activities. examples of some cell context-dependent effects of NR4As are summarized below.

Melanoma

Melanoma is a type of skin cancer that is often associated with defects in the MAPK signaling pathway and increased activity of the oncogenic BRAF [223]. The BRAF-MEK-ERK pathway regulates expression of NR4As in melanoma cells and when

NR4A1 and NR4A2 are down-regulated in melanoma cells expression of genes controlling cell proliferation, survival, and invasion is modulated [224]. This suggests that NR4As play a crucial role in melanoma tumorigenesis. Moreover, down-regulation of NR4A1 and NR4A2 up-regulates Wnt/β-catenin pathway antagonists, DACT1 and CITED1, suggesting a possible cross-talk between NR4A and β-catenin signaling pathway in melanoma cells. Ultraviolet radiation (UVR) can cause DNA damage in the form of cyclobutane pyrimidine dimers, leading to the onset of melanoma and non-melanoma skin cancer. Melanocytes synthesize melanin, which provides photoprotection, and the activation of the melanocortin-1-receptor (MC1R) signaling induces the expression of NR4A members in response to UVR [225, 226]. In addition, siRNA silencing of NR4As expression in melanocytes impairs UVR-induced DNA damage repair, suggesting that NR4As, in coordination with MC1R signaling, play an important role in UVR-induced DNA damage repair in melanoma and non-melanoma skin cancer.

Breast and prostate cancer

NR4A proteins promote cell proliferation, apoptosis and differentiation in a tissue-specific manner [71]. NR4A2 is involved in the proliferation of cancer cells, but its functional role in breast cancer has not been extensively investigated. Studies have shown that NR4A2 expression is upregulated in normal mammary epithelial cells compared with breast cancer cells and this is indirect contrast with NR4A1 levels in these tissues [227]. However, silencing of the NR4A2 gene using siRNA in mouse

mammary tumor xenografts showed a significant reduction in tumor growth compared with controls, demonstrating a role for NR4A2 in breast cancer progression [227]. In prostate cancer, NR4A2 expression was significantly higher in human prostate cancer tissues than in benign prostate tissues [228]. Silencing of NR4A2 in prostate cancer cells using siRNA in vitro decreased cell proliferation, invasion, and migration, and increased the rate of apoptosis in prostate cancer cells. Thus despite differences in the ratios of NR4A2 expression in prostate and breast cancer and non-cancer tissues, NR4A2 plays a pro-oncogenic role in both tumors.

1.2.4.3. Other inflammation diseases

Endometriosis

Safe laboratory recently confirmed that NR4A1-specific antagonist 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (DIM8-3-Cl-5-OCH3) inhibits endometriosis progression by inhibiting fibrosis in ectopic lesions [212]. NR4A1 knockout enhanced fibrosis in normal endometrial tissue (NESC) and endometriotic tissue (EESC), while CsnB (NR4A1 agonist) treatment inhibited transforming growth factor β (TGF-β)-induced fibrosis in NESC and EESC cells. Thus, NR4A1 plays a role in regulation of cell apoptosis and proliferation and also plays a role in cell metabolism. The study suggests that bis-indole-derived compounds that act as NR4A1 antagonists may be a novel non-hormonal therapy for endometriosis, and NR4A1 may be a target for non-hormonal therapy of endometriosis [212].

NF-κB inhibition

NR4A1 has anti-inflammatory properties through multiple mechanisms, including inhibition of NF-κB activation, modulation of MMP activity, and modulation of immune cell development and differentiation (rev. in [229]). In a genome-wide survey, NR4A1 was identified as the strongest inhibitor of NF-κB, a major pro-inflammatory transcription factor [230]. NR4A1 inhibits NF-κB activation by blocking binding of NF-κB to its promoter site in response to pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β), and by reducing mRNA levels and production of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8 and MCP-1 [147]. NR4A1 also inhibits the activation of the IL-2 promoter in the presence of several stimuli, preventing NF-κB from interacting with two low-affinity binding sites in its promoter. NR4A1 directly interacts with the subunit p65 of NF-κB through its C-terminal region and inhibits NF-κB activation and this also results in an interaction in which the transcriptional activation of NR4A1 is blocked [231]. Regulation of NF-κB by NR4A1 also involves elements located in the p65 C-terminal sequence, suggesting that both domains are required for optimal crosstalk between the two proteins. Phosphorylation of NR4A1 by p38α in response to LPS promotes the dissociation between this NR and p65, favoring the inflammatory response [231, 232]. Furthermore, NR4A1 prevents LPS-induced sepsis and acute liver injury through direct binding to tumor necrosis factor receptor-associated factor 6 (TRAF6) [233].

Treg cells and MMPs

NR4A1 has also been shown to regulate development of Treg cells [234, 235] and CD8+ T cells [236], and its deficiency reduces development of Ly6Clow monocytes by impairing their differentiation from myeloid dendritic precursors [237]. NR4A1 increases CCR2 levels on the surface of Ly6Chigh monocytes, promoting myocardial infiltration and differentiation into inflammatory macrophages [238]. NR4A1 plays a role in regulating MMP activity, which is important for tissue remodeling. Transgenic mice overexpressing NR4A1 express low MMP-2 levels in remodeled carotid arteries, and NR4A1 overexpression reduces MMP-2 mRNA and protein levels in VSMC (rev. in [229]). Additionally, NR4A1 may affect the activity of other MMPs by modulating TIMP-2 protease expression [239]. Thus NR4A1 has therapeutic potential in various inflammatory diseases, and its modulation may lead to the development of new treatments for these diseases.

1.3. Hypothesis, goals and objectives

Studies in our laboratory have demonstrated that several bis-indole derived compounds (CDIMs) bind NR4A1 and NR4A2 and act as highly potent anticancer agents by inhibiting NR4A1/2- mediated pro-oncogenic pathways and genes. Moreover, recent studies have characterized a series of 1,1-bis (3’ indolyl)-1-(3,5-disubstitutedphenyl) methane (DIM-3,5) analogs as dual receptor ligands that bind both NR4A1 and NR4A2 and inhibit receptor dependent pro-oncogenic gene pathways (**Figure 12**). The effects of DIM analogs in cancer cells are mimicked by many natural product-derived compounds

and we hypothesize that their mechanism of action may be due in part to their activities as NR4A1/NR4A2 ligands that act as inverse agonists.

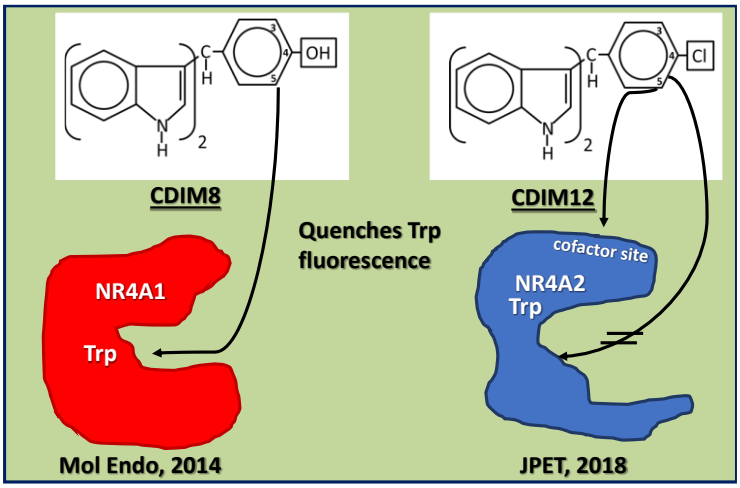


Figure 1-12. Bis-indole derivatives (CDIMs) that bind NR4A1/NR4A2. Reprinted with permission from [196, 210].

A well-known stilbene natural product called 3,5,4-trihydroxy-trans-stilbene (resveratrol) is present in foods such as blueberries, grapes, peanuts, and red wine. Like other polyphenolics, resveratrol exhibits antioxidant, anti-inflammatory, and immunomodulatory properties. Its dietary effects have been associated with prevention of cancer, cardiovascular diseases, obesity, and neurodegenerative diseases. Extensive research has been conducted on the potential therapeutic applications of resveratrol using in vitro cell culture and animal models. In cell culture models of endometriosis [240, 241], resveratrol inhibits proliferation and inflammation, including the inhibition of NFκB and other kinases [242, 243], downregulation of ESR1 [244], activation of SIRT1 [245], and reduced activities of MMP9 and MMP2 [240-246]. Resveratrol also exerts

various effects on neuronal cells, such as inhibiting brain inflammation and damage, as well as enhancing memory [247-249]. In addition, resveratrol has been extensively studied as an anticancer agent in multiple cancer cell lines and in vivo models. Resveratrol significantly inhibits cancer cell and tumor growth in osteosarcoma, melanoma, prostate, breast, kidney, renal, lung, pancreatic, and gastric cancer cells [250]. Therefore, in Aim 1 we hypothesize that the anticancer activity of resveratrol may be due, in part, to its activity as an inverse NR4A1 agonist and effects of resveratrol on lung cancer cell growth, survival and migration/invasion will be determined and compared to the effects observed after knockdown of NR4A1 by RNA interference. In addition, effects of resveratrol and NR4A1 knockdown on expression of several NR4A1-regulated pathways (mTOR) and genes (EGFR, c-Myc, Bcl-2, IDH1, Sp1, Sp4, β1-integrin, β3-integrin) will also be determined to demonstrate that resveratrol is an NR4A1 ligand that acts as an inverse agonist in lung cancer cells.

Reactive oxygen species (ROS) and oxidative stress play vital roles in maintaining cellular homeostasis and higher level of ROS trigger cell death in both non-cancer and cancer cells [251-254]. Drugs that induce ROS are being developed for cancer chemotherapy, as excessive ROS production overwhelms the cell's redox buffering capacity and leads to a cytotoxicity [253, 254]. Natural products and synthetic analogs, such as isothiocyanates, curcumin, betulinic acid, piperlongumine, and triterpenoids, are being explored as anticancer agents due to their ability to induce ROS in cancer cells and in vivo tumor models. The mechanisms behind drug-induced ROS involve targeting

mitochondria, inhibiting redox-associated intracellular pathways/genes, and depleting intracellular reductants [253, 254]. ROS activation or inactivation of genes and pathways leads to reduced cell growth, apoptosis induction, and inhibition of cell migration, invasion, and metastasis. Knockdown of NR4A1 or related genes can induce ROS, suggesting that NR4A1 ligands may possess anticancer activities through their inverse agonistic activity [171]. The natural product piperlongumine is a well-known ROS-inducing anticancer agent. In Aim 2, we will use a comparable approach to demonstrate piperlungimine displays anticancer activities through binding NR4A1.

Recent studies in this laboratory showed that two flavonoids, namely kaempferol and quercetin directly bind NR4A1 and acted as inverse agonists in Rh30 rhabdomyosarcoma cells [177]. Our previous studies showed that CDIM compounds that bound NR4A1 were highly effective as inhibitors of key pro-endometriotic pathways and genes in endometriotic cells and this includes growth, mTOR signaling and fibrosis [255]. **Aim 3** of this thesis will investigate the effects of kaempferol and quercetin as inhibitors of endometriosis in both cell culture and in vivo models (in collaboration with Sang Jun Han in Baylor College of Medicine). This research and its results will be important for developing future applications of kaempferol and quercetin as precision therapeutics for targeting endometriosis in patients which express high level of NR4A1.

Our recent discovery that the DIM-3,5- analogs bind both NR4A1 and NR4A2 may account for potent in vivo anticancer activity of several CDIMs which includes

inhibition of tumor growth in mouse orthotopic breast cancer models at doses< 1mg/kg/day [191]. These compounds bind both receptors however, their activity as novel “dual receptor” antagonists has not been determined in functional or genomic assays. Therefore, in **Aim 4** the effect of selected 3,5-disubstitutedphenyl analogs (DIM-3,5) will be determined using both functional and genomic responses and their activities will be compared to results of NR4A1 and NR4A2 knockdown alone by RNA interference. We will also use chromatin immunoprecipitation (ChIP) assays and related approaches to investigate mechanisms of coordinate regulation of some genes by both NR4A1 and NR4A2. Results of these studies in aims 1-4 will further demonstrate the potent anticancer activities of CDIM compounds and show that specific natural products cancer can be repurposed as NR4A1 ligands. This research will also identify potential clinical applications for novel synthetic NR4A1/2 ligands and repurposed agents that can be used in a precision medicine approach for treating cancer patients expressing these orphan nuclear receptors.

CHAPTER II

RESVERATROL BINDS NUCLEAR RECEPTOR 4A1 (NR4A1) AND ACTS AS AN NR4A1 ANTAGONIST IN LUNG CANCER CELLS[*]

2.1. Introduction

Dietary polyphenolics produced in vegetables, fruits, and nuts have been associated with multiple beneficial health effects, including longer lifespans and other age-related diseases [256-258]. Polyphenols associated with these health benefits include phenolics acids, coumarins, flavonoids, lignans, and stilbenes including 3,4',5-trihydroxy-trans-stilbene (resveratrol), which is enriched in foods such as blueberries, grapes, peanuts, and red wine and exhibits prototypical polyphenolic health benifits [250, 259-262]. Resveratrol has been extensively investigated as a therapeutic agent for treatment of multiple diseases in both in vitro cell culture and animal models. Resveratrol inhibits proliferation and inflammation in cell culture models of endometriosis [240, 241], and this includes inhibition of nuclear factor kB (NFkB) and other kinases [242, 243], downregulation of estrogen receptor (ESR1) [244], activation of NAD-dependent de-acetylase sirtuin-1 (SIRT1) [245], and decreased matrix metallopeptidase 9 (MMP9) and MMP2 activities [246]. Resveratrol also has multiple effects on neuronal cells in culture and in vivo, and this includes inhibition of brain inflammation, damage, and enhanced memory [247-249]. For example, in rat brain resveratrol upregulates Sirt1/microRNA-Sp

[*] **Reprinted with permission from: Zhang, L., Martin, G., Mohankumar, K., Hampton, J. T., Liu, W. R., & Safe, S. (2022). Resveratrol Binds Nuclear Receptor 4A1 (NR4A1) and Acts as an NR4A1 Antagonist in Lung Cancer Cells. *Molecular Pharmacology*, *102*(2), 80-91.**

to protect against ischemia [247], whereas resveratrol protection in neonatal hypoxic-ischemic brain injury involves SIRT1-regulated inhibition of high mobility group box 1 protein (HMGB1) and downstream NFkB signaling [248]. Moreover, induction of inflammation in BV2 cells enhances toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), NFkB, and multiple cytokines, and these responses are inhibited by resveratrol [248]. Resveratrol also activates a SIRT1/NFkB to protect against sevoflurane-induced cognitive impairment in mice [249].

Resveratrol is a highly effective inhibitor of cancer cell and tumor growth, migration, and invasion in multiple cell lines (rev. in [250]), and in most of these cell lines this was accompanied by altered regulation of pathways/gene products associated with these anticancer activities. The results of many studies on the anticancer activities of resveratrol have been variable and cell context-specific; however, some pathways and genes such as resveratrol-dependent inhibition of the mammalian target of rapamycin (mTOR) pathway have been reported in many different cancer cell lines [263-272].

Research in our laboratory has been focused on the orphan nuclear receptor 4A1 (NR4A1, Nur77) and its functions in cancer and noncancer cell lines and animal models [267]. Nr4a1 and other members of this family (Nr4a2 and Nr4a3) are immediate early genes that are induced by diverse stressors to maintain cellular homeostasis, and NR4A1 is overexpressed in solid tumor-derived cancers and other inflammatory diseases [273, 274]. Results primarily of NR4A1 knockdown studies show that this receptor regulates cancer cell growth, survival, migration, and invasion, and this includes mTOR signaling pathways on multiple cancer cell lines. NR4A1 regulates prosurvival and growth

promoting genes such as epi- dermal growth factor receptor, survivin, and B-cell lymphoma 2 (Bcl-2), as well as β1-integrin and other integrins in many different cancer cell lines. Isocitrate dehydrogenase-1 (Idh-1) and thioredoxin domain containing 5 (Txndc5) are also NR4A1-regulated genes in solid tumor-derived cell lines, and these genes serve to maintain high reductant levels in cancer cells (rev. in [159]). Bis-indole derived com- pounds (CDIMs) have been characterized as ligands that bind NR4A1 and act as NR4A1 antagonists that inhibit cancer cell growth, survival, migration, and invasion [159, 210]. CDIM-NR4A1 antagonists also inhibit mTOR in lung and other cancer cell lines [159, 160, 210], and many of the effects of these NR4A1 antagonists have also been observed for resveratrol [160, 250, 259, 270, 275]. We hypothesize that one of the underlying mechanisms of action of resveratrol in cancer cells is that of an NR4A1 ligand that acts as an antagonist, and this study demonstrates for the first time that resveratrol is an NR4A1 ligand.

2.2. Materials and methods

2.2.1. *Ligand – receptor binding assays*

Isothermal titration calorimetry (ITC) was used to determine the ligand binding constant (Kd) to NR4A1 utilizing an Affinity ITC (TA Instruments, New Castle, DE). Briefly, the experimental setup was as follows. The ITC sample cell contained 250 μl of NR4A1 protein [ligand binding domain (LBD)] at a concentration of 20 μmol/l in buffer containing 20 mmol sodium phosphate/l (pH 7.4), 5% glycerol, and 1.0% ethanol. The

ligand titrant was prepared in the same buffer as above at a ligand concentration of 66.6 μmol/l. The ligand titration into protein was performed at 25 °C with a stir rate of 125 rpm. Each ligand injection volume was 5 μl followed by 200 seconds to measure the total heat flow required to maintain constant temperature. A total of 20 injections were done for each ligand/NR4A1 combination. Each ligand titration into protein experiment was repeated for a total of three separate and independent experiments to generate the curves shown in the figure. In a separate set of injections, the same ligand was injected into buffer only (no protein) to determine heat flow as a result of ligand dilution into buffer. The ligand/buffer values were subtracted from the ligand/ protein values prior to data analysis using the Affinity ITC manufacturer-supplied data analysis software package. Sigmoidal curve fitting was performed using the Affinity ITC manufacturer-supplied data analysis software package to determine the following binding parameters: Kd, the equilibrium binding dissociation constant (μmol/l); n, the equilibrium ligand-to-protein binding stoichiometry (mol ligand per mol NR4A1); and ΔG, the equilibrium free energy of ligand binding (kJ/mol). The resulting data are plotted as heat flow/area data (μJ) versus the cumulative resveratrol concentration μmol/l) present in the sample cell. Statistical analysis of the triplicate data was performed utilizing SigmaPlot 14.5 (Systat Software, Inc.) to determine the parameter mean (Kd, n, ΔG) and standard deviation. In addition, we also used a direct binding assay by determining the loss of fluorescence of a tryptophan residue in the LBD as previously described [210].

2.2.2. Computation-based molecular modeling

Molecular modeling studies were conducted using Maestro (Schrödinger Release 2020-1, Schrödinger, LLC, New York, NY, 2020). The version of Maestro used for these studies is licensed to the Laboratory for Molecular Simulation, a Texas A&M University core user facility for molecular modeling and is associated with the Texas A&M University High Performance Research Computing facility. All Maestro-associated applications were accessed via the graphical user interface (GUI) VNC interactive application through the HPRC Ada OnDemand portal. The crystal structure coordinates for human orphan nuclear receptor NR4A1 ligand binding domain (LBD) [158] were downloaded from the Protein Data Bank (https://www.rcsb.org; PDB ID 3V3Q). The human NR4A1 LBD crystal structure was prepared for ligand docking utilizing the Maestro Protein Preparation Wizard; restrained minimization of the protein structure was performed utilizing the OPLS3e force field. Each ligand (resveratrol or DIM-3,5-Cl2) three-dimensional structure was prepared for docking utilizing the Maestro LigPrep, again using the OPLS3e force field. Maestro Glide [276, 277] was used with the default settings to dock each prepared ligand to the prepared protein, predict the lowest energy ligand binding orientation, and calculate the predicted binding energy in units of kcal/mol.

2.2.3. Cell culture, reagents, and antibodies

H460 and H1299 lung cancer cells are purchased from American Type Culture Collection (Manassas, VA). Both cell lines were derived from male patients with

nonsmall cell lung cancer (H1299) or large cell lung cancer (H460). Cells are cultured in RPMI1640 medium with 10% FBS at 37 °C in the presence of 5% CO2. The details of antibodies used for Western blots and for chromatin immunoprecipitation (ChIP) assays are summarized in Supplemental Table 1.

2.2.4. Cell proliferation assay

Cell proliferation was investigated using XTT Cell Viability Kit (Cell Signaling Biotechnology) according to the manufacturer's instructions. Cells (1.5×10^4/well) were plated in 100 μl of plating medium (as above) on 96-well plates and allowed to attach for 24 hours. The medium was then changed to RPMI 1640 containing 2.5% charcoal-stripped FBS, and either vehicle DMSO or different concentrations of compounds in DMSO were added. After 24, 48, and 72 hours of culture, 25 μl of XTT reaction solution (sodium 30 -[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy- 6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate (mixed in proportion 50:1) were added to the each well. The optical density was read at 490 nm wavelength in a plate reader after incubation for 4 hours. All determinations were replicated in at least three separate experiments.

2.2.5. Transfection and luciferase assay

Cells were plated on 12-well plates at 5×10^4/well in RPMI 1640 medium supplemented with 2.5% charcoal-stripped FBS. After 24-hour growth, various amounts of DNA [i.e.,

UASx5-Luc (400 ng), GAL4-NR4A1 (50 ng) and b-gal (50 ng)] were cotransfected into each well by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with plating media (as above) containing either solvent (DMSO) or the indicated concentration of compound for 18 hours. Cells were then lysed using a freeze-thaw protocol, and 30 μl of cell extract was used for luciferase and b-gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and b-gal activities. Luciferase activity values were normalized against corresponding β-gal activity values as well as protein concentrations determined by Bradford assay.

2.2.6 Annexin V staining assay

Annexin V staining assay was performed using Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (Invitrogen, Carlsbad, CA). Briefly, cells were seeded in 6-well plates followed by various drug treatments. The cells were then washed with ice cold PBS, and 5 μl Alexa Fluor 488 Annexin V with 100 μg/ml PI (as per the manufacturer instructions) were added to the cells and incubated for 15 min. The cells were determined by Accuri flow cytometer.

2.2.7. Boyden chamber invasion zssay and scratch migration assay

Attached cells (2.0×10^5) were treated with DMSO or with different concentrations of resveratrol in medium supplemented with 2.5% charcoal stripped FBS for 24 hours or

transfected with different small interfering RNAs (siRNAs) with RNA iMax transfection for 72 hours as manufacturer's protocol. Then, for Boyden chamber invasion assay, 1.0 × 10^5 cells from each treatment condition were allowed to invade through the Boyden Chamber for 48 hours. Cells that invaded into the Boyden Chamber were fixed using formaldehyde, stained, and then counted. For scratch migration assay, cells were grown to 90% confluency in 6-well plates, then scratched with a 200 μl sterile pipette tip and washed with PBS to remove detached cells from the plates. Cells were kept in incubator with DMSO or indicated treatments for 48 hours. After 48 hours, cells were fixed with 4% formaldehyde and stained with crystal violate solution. The wound gap was observed under AMG EVOS fl microscope. At least 3 replicates were performed for each treatment group.

2.2.8. Western blot analysis

Cells (3.0 × 10^5) were seeded on 6-well plates, and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer RIPA (Thermo Scientific, Waltham, MA) that contained protease and phosphatase inhibitors (GenDEPOT, Baker, TX). The total protein in the lysates was quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel. The proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane, then blocked for an hour using 5% skimmed milk. The membranes were then incubated with primary antibody for overnight at 4 °C. It was then washed with Tris-buffered saline and Polysorbate 20 and incubated with horseradish peroxidase-

linked secondary antibody for 1 hour at room temperature. The membranes were further washed with Tris-buffered saline and treated with Immobilon western chemiluminescence horseradish peroxidase-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR). Protein levels in various treatment groups were normalized to b-actin.

2.2.9. Transfection and small interfering RNAs

For RNA interference experiment, cells were seeded on 6-well plates at 3×10^5/well then allowed 24 h to attach and grow. Then, they were transfected with siRNA of 100 nmol each/well for 6-well plates using 6.5 μl/well RNA iMax transfection reagent for 72 hours. siRNAs targeting NR4A1 (siNR4A1), Sp1 (siSp1), and Sp4 (siSp4) were purchased from Sigma-Aldrich. Negative Control Ig L2 siRNA were purchased from Qiagen. The oligonucleotides used were as follows:

siNR4A1_1, SASI_Hs02_00333289

siNR4A1_2, SASI_Hs02_00333290

siSp1_1: SASI_HS01-00070994

siSp1_2: SASI_Hs02_00333289

siSp4_1: SASI_HS01-00114420

siSp4_2: SASI_HS01-00114421.

2.2.10. ChIP assay

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin im- munoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. All cells (3×10^7) were treated with DMSO or indicated concentration of resveratrol for 3 hours. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (200–1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG (Cell signaling), NR4A1 (Abcam), Sp1 (Abcam), Sp4 (Santa Cruz), or RNA polymerase II (pol II; GeneTex) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by polymerase chain reaction (PCR) amplification. The primers for detection of the β1 integrin promoter region were 5 -TCA CCA CCC TTC GTG ACA C-3(sense) and 5 -GAG ATC CTG CAT CTC GGA AG-3(antisense). PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (EtBr).

2.2.11. Real time-PCR

RNA was isolated using Qiagen RNeasy Mini kit (Irvine, CA). Quantification of mRNA (β1-integrin) was performed using Bio-Rad iTaq Universal SYBR Green 1-Step Kit

(Richmond, CA) using the manufacturer's protocol with real-time PCR. Human GAPDH mRNA was used as a control to determine relative mRNA ex- pression. The primers for detection of the β1 integrin mRNA were 5- GAA GGG CGT GTT GGT AGA CA-3 (Forward) and 5-GTT GCA CTC ACA CAC ACG AC-3 (Reverse).

2.2.12. Statistical analysis

Each assay was performed in triplicate and the results were presented as means with S.D. The statistical significance of differences between the treatment groups was determined by Dunnett's multiple comparison test in ordinary one-way ANOVA. Analysis of Western blotting was done using ImageJ (1.53K) soft- ware. GraphPad Prism 8 (Version 8.4.3) software was used for analysis of variance and determined statistical significance. Data with a P value of less than 0.05 were considered statistically significant and indicated with "*" in figures.

2.3. Results

2.3.1. Binding and transactivation

Based on the similarities between the effects of resveratrol and NR4A1 antagonists on solid tumor derived cancer cells, we initially investigated the binding of resveratrol (Fig. 2-1A) to the ligand binding domain of human NR4A1 using an isothermal titration calorimetry (ITC) assay procedure. The results showed that resveratrol bound to NR4A1 with a calculated KD value of 2.4 ± 0.7 μM (S.D., 3 determinations) and a DG value of -

32.2 ± 0.8 kJ/mol (Fig. 2-1B). The stoichiometry of binding (n) is 0.82 mol ligand bound/mol protein. The direct interaction of resveratrol with NR4A1 was also confirmed in a fluorescence quenching assay of a Trp in the NR4A1 binding pocket [210] and the KD value was 1.4 μM. We also used a computer modeling approach and compared the predictive interactions of resveratrol in the ligand binding pocket of NR4A1 (Fig. 2-1C) to that observed for the bis-indole NR4A1 ligand bis(30-indolyl)-1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl2) [274] (Fig. 2-1D). The simulations predicted that both compounds interact with common amino acids side chains Ser110, Glu114, Arg184, and Thr236; in addition, they also interacted uniquely with amino acid side chains Leu113, Leu239, and Ile260 (resveratrol), and Arg232 (DIM-3,5-Cl2), demonstrating some ligand structure-dependent differences in binding of resveratrol and DIM-3,5-Cl2 to NR4A1.

The activity of resveratrol as an NR4A1 ligand was confirmed in transactivation assays in H460 and H1299 lung cancer cells transfected with a yeast GAL4-NR4A1 chimera construct and a UAS-luc reported gene containing 5 tandem yeast GAL4 binding elements. Resveratrol decreased transactivation in both cell lines (Fig. 2-1, E and F), indicating NR4A1 antagonist activity, which has previously been observed for the CDIM/NR4A1 ligands in lung and other cancer cell lines [159, 160, 278, 279].

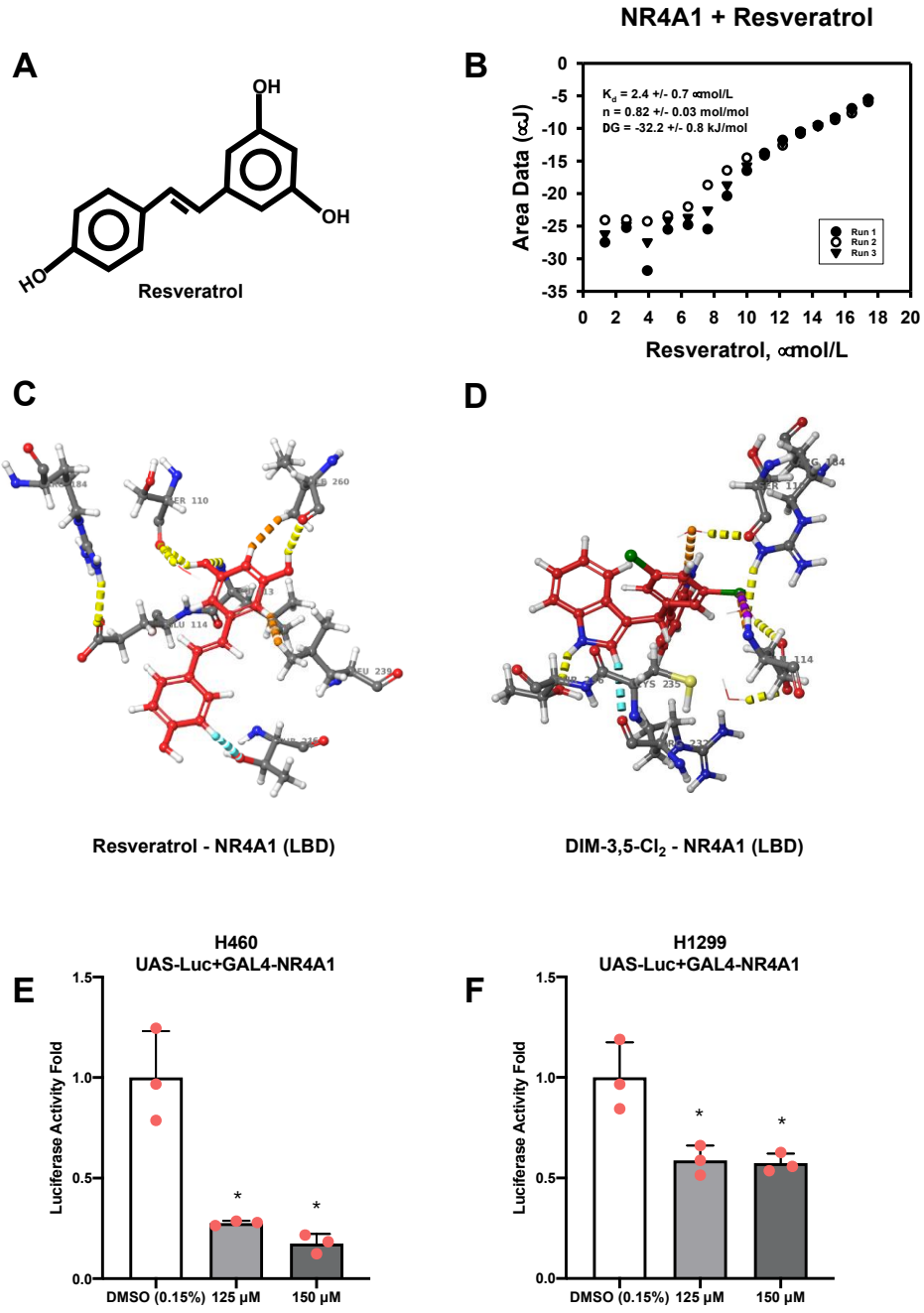


Figure 2-1. Resveratrol as an NR4A1 ligand. (A) Structure of resveratrol B. Binding of resveratrol to NR4A1 (LBD) by isothermal titration calorimetry (ITC) (B) as outlined in the Methods. Molecular modeling of the interaction of resveratrol (C) and 1,1-bis(30-indolyl)-1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl2) (D) with NR4A1 (LBD) was carried out using Maestro and crystal structure coordinates for the human orphan nuclear receptor NR4A1 ligand binding domain as outlined in the Methods. Effects of resveratrol on luciferase activity in H460 (E) and H1299 (F) cells transfected with GAL4- NR4A1 and UAS-luc as outlined in the Methods. Results are expressed as means ± S.D. for at least 3 replicate determinations for each treatment group and significant (P < 0.05) effects compared with control are indicated (*). The ITC binding assay was repeated (3X) and the means KD and DG values ± S.D. are indicated in panel 1B.

2.3.2. Resveratrol and NR4A1 knockdown inhibit lung cancer cell growth, survival, migration, and invasion.

Treatment of lung cancer cells with resveratrol decreased H460 and H1299 cell growth by approximately 40%–50% (Fig. 2-2, A and B) and also decreased NR4A1 protein expression (Fig. 2-2C). Knockdown of NR4A1 also decreased lung cancer cell growth by 40%–65% (Fig. 2-2, D and E), and NR4A1 protein (Fig. 2-2F) and levels of NR4A1 protein are quantitated (Supplemental Fig. 1A). Cell growth inhibition was observed over a range of concentrations (50–150 μM) and previous studies in lung cancer cells used concentrations of 100 or 200 μM to investigate effects of resveratrol on multiple endpoints [270, 275]. Based on preliminary studies we used two concentrations (125 and 150 μM) of resveratrol, which changed most pathways and levels of gene products investigated in this study. We also observed that resveratrol (125 and 150 μM) induced markers of apoptosis (PARP and caspase-3 cleavage; Bcl-2 downregulation) (Fig. 2-2G). Similar results were observed after knockdown of NR4A1 (Fig. 2-2H), and quantitation of the western blots (G and H) are summarized in Supplemental Fig. S1B and S1C. Resveratrol (125 and 150 μM) also induced Annexin V staining in H460 and H1299 cells (by >12-fold) (Fig. 2-2I). In addition, we also observed that resveratrol inhibited cell migration in a scratch assay by >45% at the high dose, and similar results were observed after NR4A1 knockdown (approximately 25% inhibition) (Fig. 2-3, A and B). Resveratrol (125 and 150 μM) inhibited cell invasion by 75% in a Boyden chamber assay by >25% after receptor knockdown (Fig. 2-3, C and D). These results demonstrate that the functional inhibitory effects of resveratrol on H460 and H1299 cell growth,

survival, migration, and invasion mimic those obtained after knockdown of NR4A1, suggesting that the anticancer activity of resveratrol is due, in part, to its activity as an NR4A1 ligand.

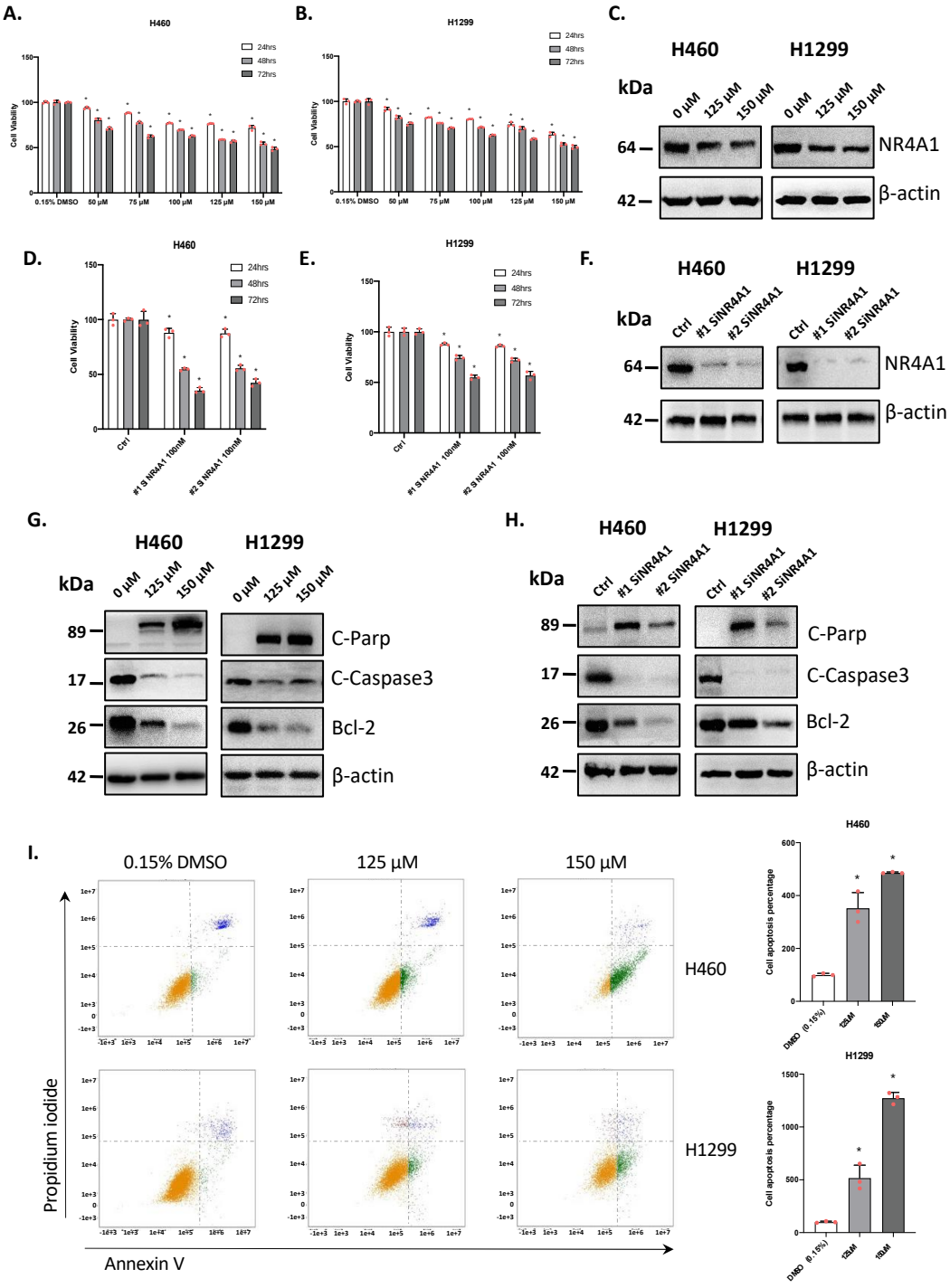


Figure 2-1. Resveratrol and NR4A1 knockdown (siNR4A1) inhibit growth and induce apoptosis in H460 and H1299 cells. H460 (A) and H1299 (B) cells were treated with resveratrol for up to 72 hours, and effects on cell proliferation and NR4A1 protein expression (C) were determined using an XTT assay. H460 and H1299 cells were transfected with siNR4A1 (2 oligonucleotides), and effects on proliferation of H460 (D) and H1299 (E) cells and NR4A1 protein expression (F) were determined. (G) H460 and H1299 cells were treated with resveratrol, or

whole cell lysates were analyzed by western blots, and bands were quantitated. H460 and H1299 cells were transfected with siNR4A1 (H), whole cell lysates were analyzed by western blots, and bands were quantitated. (I) H460 and H1299 cells were treated with resveratrol, and effects on Annexin V staining were determined as outlined in the Methods. Results are expressed as means ± S.D. for at least 3 separate determinations for each treatment group, and significant ($P < 0.05$) changes compared with control (DMSO) are indicated. Calculations of changes in intensity of protein bands are also normalized to the b-actin loading control for each treatment group. Quantitation of blots in 2-2C/2F, 2-2G, and 2-2H are summarized in Supplemental Fig. 2-1A, 2-B, and 2-C, respectively.

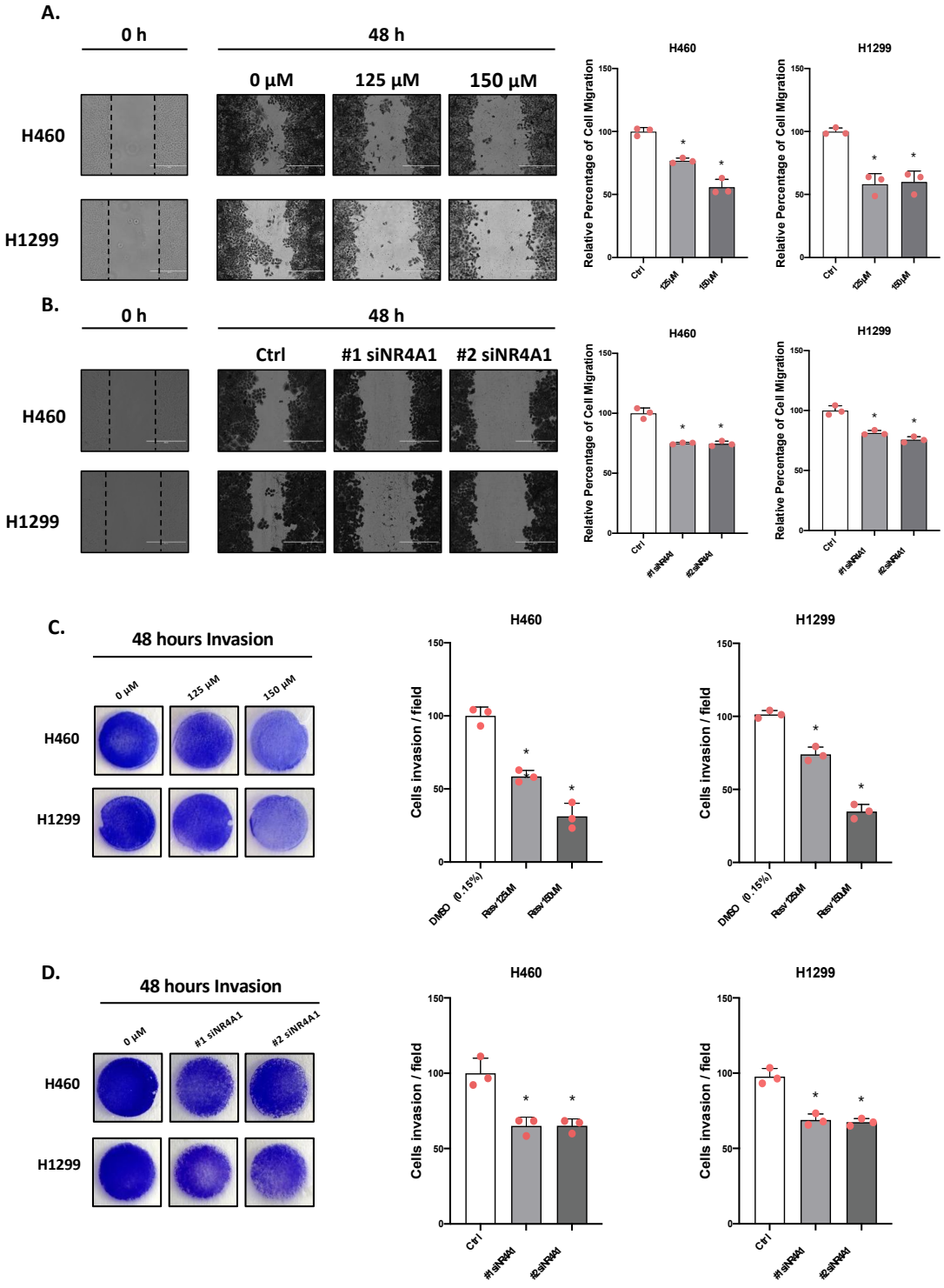


Figure 2-2. Resveratrol inhibits cell migration and invasion. (A) H460 and H1299 cells were treated with resveratrol (A) or transfected with siNR4A1 oligonucleotides (B), and effects on cell migration were determined in scratch assays. Cells were treated with resveratrol (C) or transfected with siNR4A1 oligonucleotides (D), and

effects on cell invasion were determined in a Boyden chamber assay. Results of the effects of resveratrol (A and C) and knockdown (B and D) have been quantitated and appear in the same panels (right side). Quantitative results are expressed as means ± S.D. for at least 3 separate determinations per treatment group, and significant ($P < 0.05$) inhibition is indicated (*). Resveratrol (125 and 150 μM) decreased migration ability in H460 cells by 23.22% with a 95% CI of 13.37%–33.06% and 44.19% with a 95% CI of 34.34%–54.04%, respectively. Resveratrol (125 and 150 μM) decreased cell migration ability in H1299 cells by 41.76% with a 95% CI of 25.16%–58.35% and 40.10% with a 95% CI of 23.50%–56.70%, respectively. Resveratrol (125 and 150 μM) decreased invasion ability in H460 cells by 41.43% with a 95% CI of 25.94%–56.92% and 68.78% with a 95% CI of 53.29%–84.27%, respectively. Resveratrol (125 and 150 μM) decreased invasion ability in H1299 cells by 27.30% with a 95% CI of 17.34%–37.26% and 66.46% with a 95% CI of 56.50%–76.41%, respectively.

2.3.3. Resveratrol and NR4A1 knockdown modulate expression of several gene products (proteins) and mRNAs in common.

Previous studies in multiple solid tumor derived cell lines have identified several NR4A1-regulated genes and proteins, and they include thioredoxin containing domain 5 (TXNDC5), isocitrate dehydrogenase-1 (IDH-1), and inhibition of phospho-AMP-activated protein kinase-α (p-AMPKα) [72, 160, 210, 280]. Treatment of H460 and H1299 cells with resveratrol (125 and 150 μM) decreased expression of TXNDC5 and IDH-1 and induced p-AMPKα proteins (Fig. 2-4, A and B), and similar results were obtained in H460 and H1299 cells transfected with oligonucleotides targeted to NR4A1 (#1 siNR4A1, #2 siNR4A1) (Fig. 2-4, C and D), confirming comparable effects of resveratrol and NR4A1 knockdown. Previous studies show that resveratrol and CDIM/NR4A1 antagonists inhibit mTOR and downstream pathways, and results in Fig. 2-5, A and B (quantitation) show that resveratrol (125 and 150 μM) inhibited phosphorylation of mTOR and the downstream kinases S6RP and 4E-BP1 in H460 and H1299 cells.

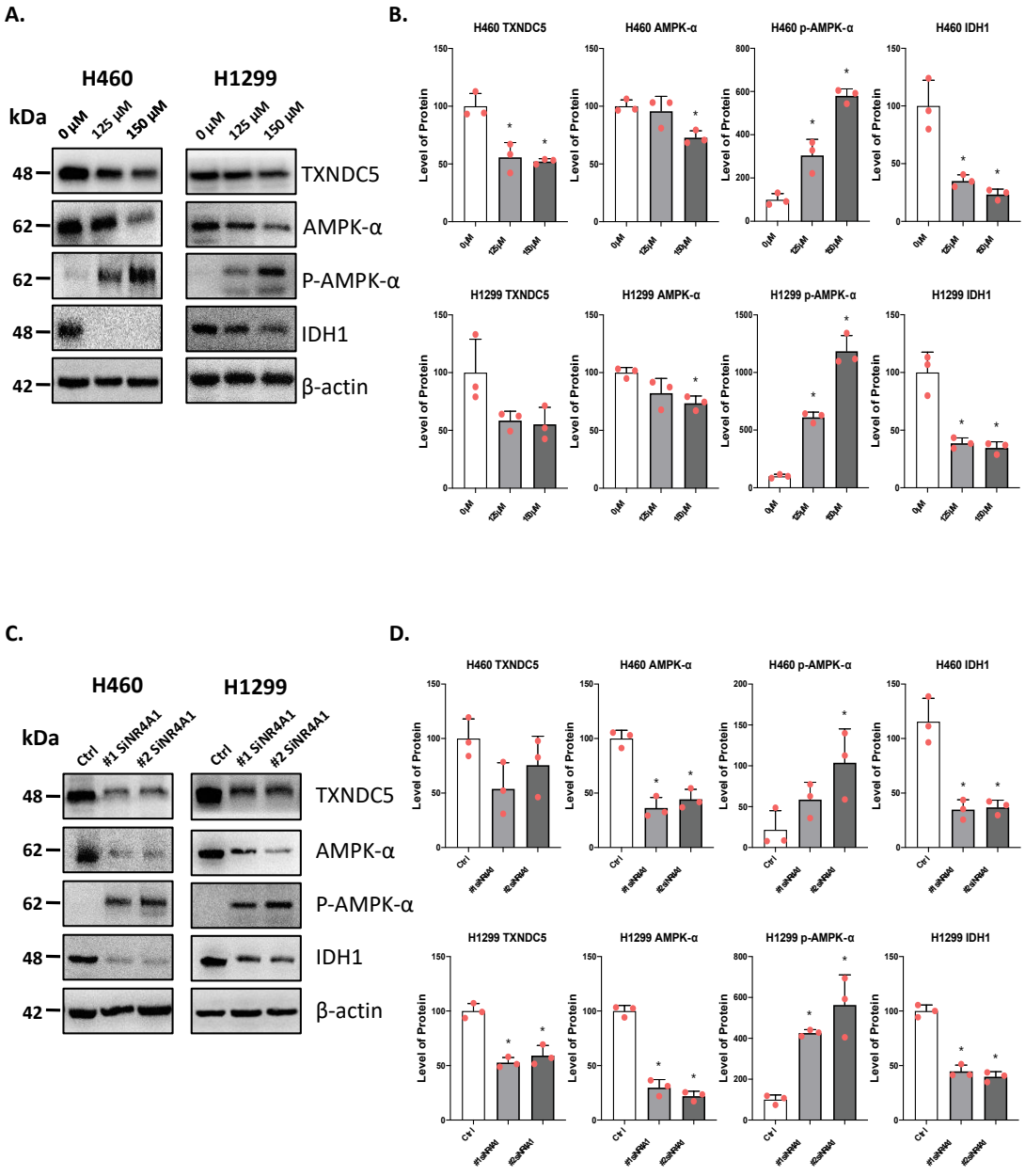


Figure 2-1. Effects of resveratrol and NR4A1 knockdown on selected NR4A1-regulated gene products. H460 and H1299 cells were treated with resveratrol (A; B-quantitation of bands in A) or transfected with siNR4A1 oligonucleotides (C; D-quantitation of bands in C), and whole cell lysates were analyzed by western blots as outlined in the Methods. Quantitative results (B and D) are expressed as means ± S.D. for at least 3 separate gels per treatment group, and significantly ($P < 0.05$) induced changes in band densities compared with CTL (DMSO or empty vector) are indicated (*). Levels for all proteins were normalized to β-actin.

These results are consistent with activation of AMPK by resveratrol (Fig. 2-4); however, in this study resveratrol also downregulated the kinase proteins (mTOR, S6RP, and 4E-BP1), and this would also contribute to their decreased phosphorylation. The effects of NR4A1 knockdown in H460 and H1299 cells (Fig. 2-5, C and D) also resulted in decreased expression of mTOR, S6RP, and 4E-BP1 and their phosphorylated forms, and thus resembled effects observed in cells treated with resveratrol. NR4A1 not only directly binds promoter DNA, but also acts as a nuclear cofactor that activates expression of several genes and proteins in cancer cells through protein-protein interactions with Sp1 or Sp4 bound to GC-rich promoter sites [82, 159, 160, 185, 281-283]. This is commonly observed for other nuclear receptors that act as ligand-dependent nuclear cofactors [86]. β1-integrin is regulated by NR4A1/Sp1 and NR4A1/Sp4 in rhabdomyosarcoma, breast, colon, and pancreatic cancer cells, and interactions with Sp1 or Sp4 are cell context dependent [160, 185, 281-283] . Fig. 2-6A illustrates that treatment of H460 or H1299 cells with resveratrol or knockdown of NR4A1 by RNA interference (RNAi) decreases levels of β1-integrin protein (quantitation in Supplemental Fig. 2-1D), and resveratrol also decreased β1-integrin mRNA levels (Fig. 2-6B), confirming that β1-integrin is an NR4A1-regulated gene. Knockdown of Sp1 in H460 and H1299 cells decreased Sp1 but only minimally affected Sp4 expression, and this was accompanied by decreased levels of β1-integrin protein, confirming a role for NR4A1/Sp1 (Fig. 2-6C). In contrast, knockdown of Sp4 by RNAi decreases expression of both Sp1 and Sp4 proteins and also β1-integrin protein (Fig. 2-6D).

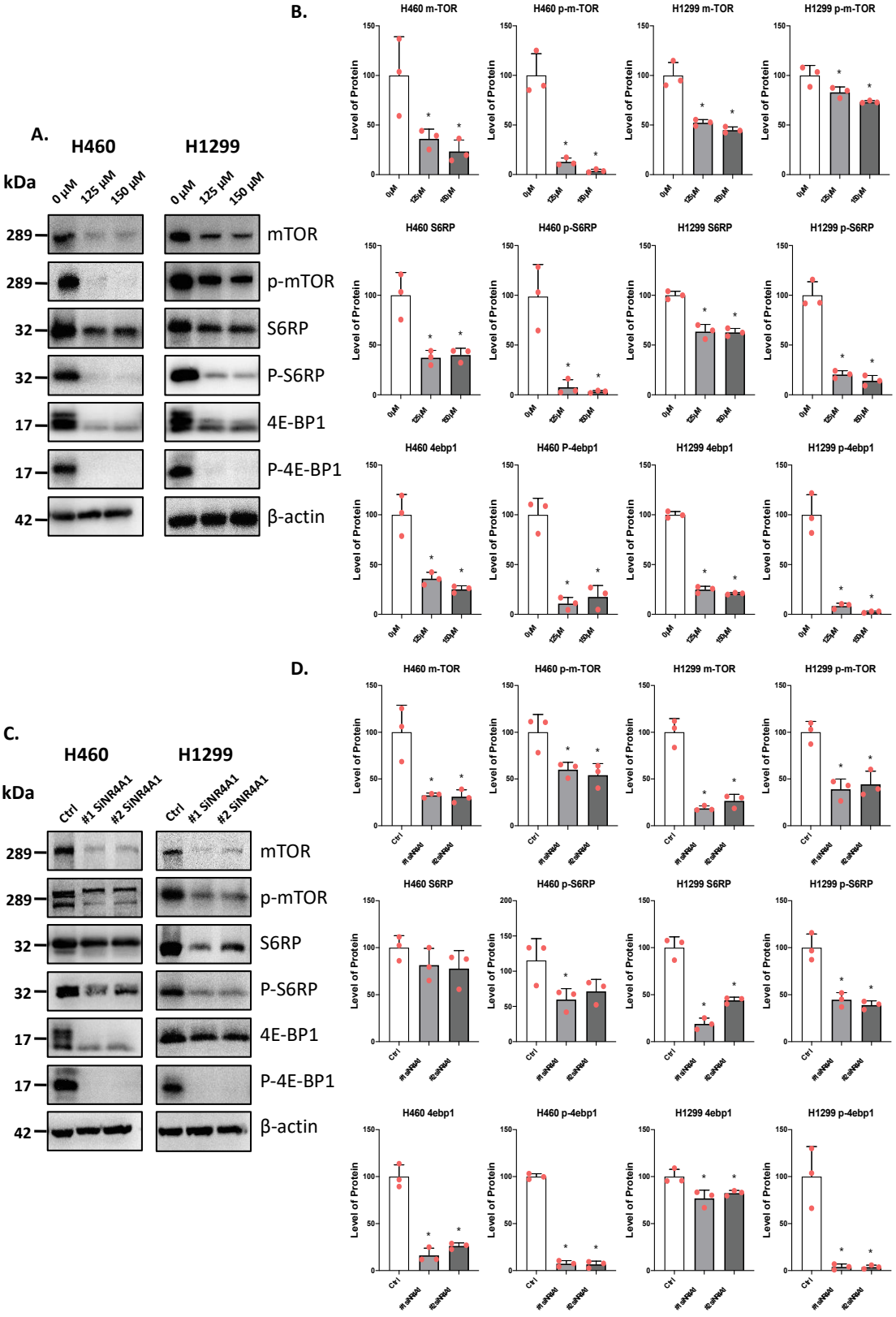

A.
H460
H1299
kDa
0 μM
125 μM
150 μM
289
mTOR
289
p-mTOR
32
S6RP
32
P-S6RP
17
4E-BP1
17
P-4E-BP1
42
β-actin
B.
H460 m-TOR
H460 p-m-TOR
H1299 m-TOR
H1299 p-m-TOR
H460 S6RP
H460 p-S6RP
H1299 S6RP
H1299 p-S6RP
H460 4ebp1
H460 P-4ebp1
H1299 4ebp1
H1299 p-4ebp1
Level of Protein
C.
H460
H1299
kDa
Ctrl
#1 SiNR4A1
#2 SiNR4A1
289
mTOR
289
p-mTOR
32
S6RP
32
P-S6RP
17
4E-BP1
17
P-4E-BP1
42
β-actin
D.
H460 m-TOR
H460 p-m-TOR
H1299 m-TOR
H1299 p-m-TOR
H460 S6RP
H460 p-S6RP
H1299 S6RP
H1299 p-S6RP
H460 4ebp1
H460 p-4ebp1
H1299 4ebp1
H1299 p-4ebp1
Level of Protein
Ctrl
#1 siRNAi
#2 siRNAi

Figure 2-2. Effects of resveratrol and siNR4A1 on mTOR signaling. H460 and H1299 cells were treated with resveratrol (A; B-quantitation of bands in A) or transfected with siNR4A1 oligonucleotides (C; D, quantitation of bands in C), and whole cell lysates were analyzed by western blots as outlined in the Methods. Results (B and D) are expressed as means ± S.D. for at least 3 separate gels per treatment group, and significantly ($P < 0.05$) induced changes in band intensities compared with CTL (DMSO or empty vector) are indicated (*). Levels for all proteins were normalized to β-actin.

Thus, it is not possible to demonstrate unambiguously whether NR4A1/Sp4 regulates expression of β1-integrin, and quantitation of these data are illustrated in Supplemental Fig. 1, E and F. ChIP analysis shows that in H460 cells treated with DMSO (control) or resveratrol that NR4A1, Sp1, and Sp4 were associated with the GC-rich promoter region of the β1-integrin gene, and treatment with resveratrol resulted in some loss of NR4A1, Sp1, and Sp4 binding (Fig. 2-6E) to the promoter. Similar results were observed in previous studies with CDIM/NR4A1 antagonist [160, 185, 281-283], demonstrating that the anticancer activity of resveratrol in H460 and H1299 lung cancer cells is due, in part, to the activity of resveratrol as an NR4A1 antagonist.

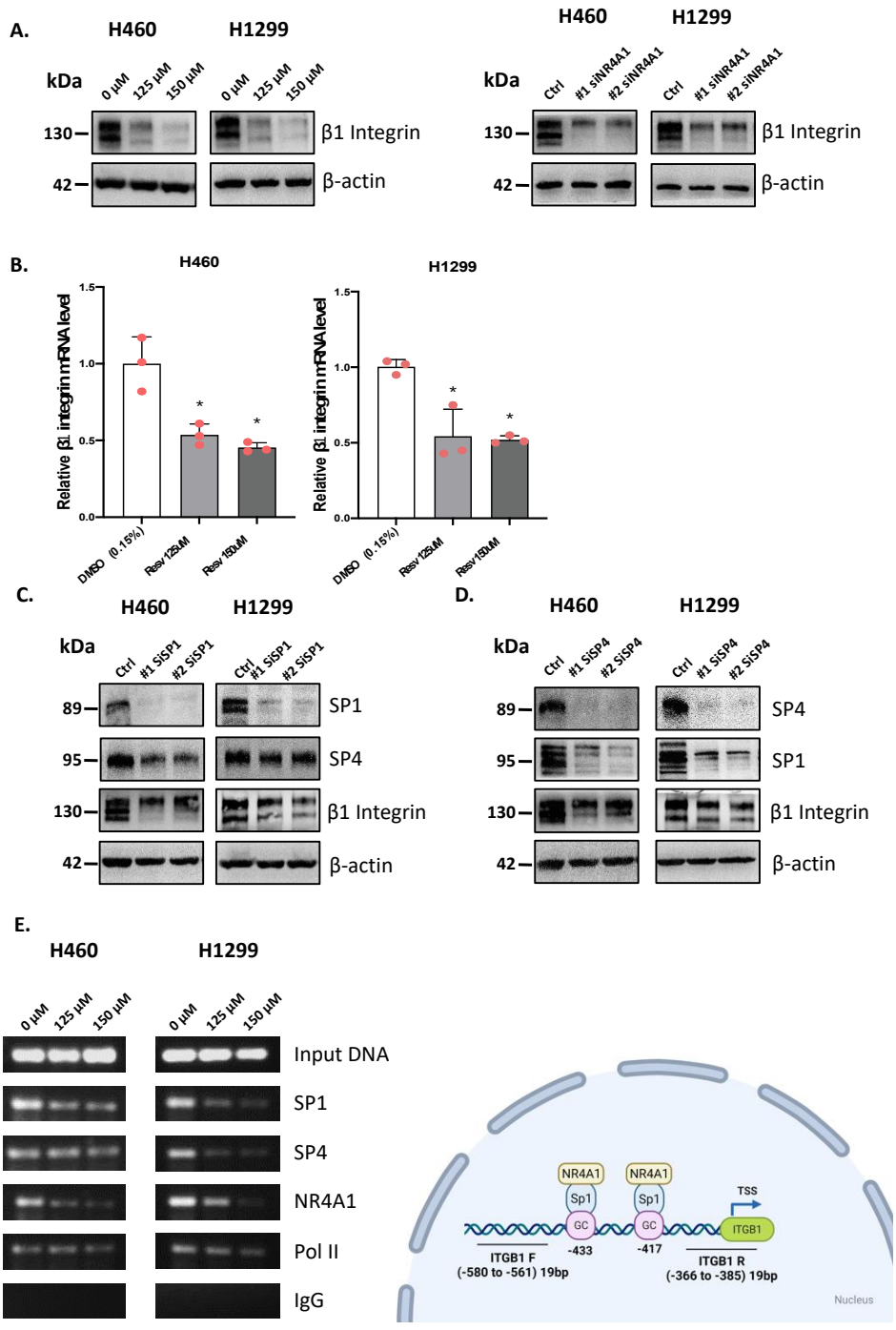


Figure 2-3. Mechanism of β1-integrin regulation by resveratrol. H460 and H1299 cells were treated with resveratrol for 24 hours, and whole cell lysates were analyzed by western blots or transfected with siNR4A1 oligonucleotides (A). (B) H460 and H1299 cells were treated with resveratrol for 24 hours, and β1-integrin mRNA levels were determined by real-time PCR as outlined in the Methods. H460 and H1299 cells were transfected with oligonucleotides that target Sp1 (siSp1) (C) or Sp4 (siSp4) (D) expression, and whole cell lysates were analyzed by western blots. (E) Effects of resveratrol on interactions of NR4A1 Sp1, Sp4, and pol II with the GC-rich region

of the β1-integrin promoter were determined in a ChIP assay as outlined in the Methods. Quantitative results (B) are means ± S.D. for at least 3 replicate determinations, and significant ($P < 0.05$) changes relative to untreated control values are given (*). Quantitation of western blots in A, C, and D are summarized in Supplemental Fig. 2-1D, E, and F, respectively.

2.4. Discussion

Polyphenolic compounds, including resveratrol, are enriched in diets containing fruits, nuts, and vegetables, and their consumption is associated with numerous health benefits, including longer lifespans and protection from aging- related and inflammatory diseases including cancer [250, 256-262]. These compounds act as antioxidant, anti-inflammatory, and antiviral agents and also regulate multiple pathways and genes that contribute to diverse disease states and serve as an important class of dietary chemo-preventive agents. Resveratrol has been extensively investigated in preclinical cell culture and in vivo models and exhibits impressive cancer chemotherapeutic properties that have been attributed to the effects of this compound on expression of multiple genes. This also includes activation of AMPK and subsequent inhibition of mTOR signaling, as well as the effects of resveratrol on activation of SIRT1 and other histone and nonhistone deacetylase, which are two pathways linked to the chemotherapeutic effects of resveratrol [259, 263-272]. Results of preliminary studies showed that effects of resveratrol on SIRT1 expression in H460 and H1299 cells were cell context dependent and highly variable (data not shown).

Despite the remarkable activities of resveratrol, the effects of this compound in human clinical trials have not matched the promise of results from preclinical cell culture and

animal models of disease [284-287]. Although resveratrol is generally well tolerated and provides some indications of benefits, poor bioavailability has been a problem, and detrimental effects have been observed for some cancers. For example, resveratrol inhibited several kinases in models of multiple myeloma [288]; however, treatment of drug-resistant multiple patients with myeloma with resveratrol resulted in several toxic side effects including renal failure. It is also possible that the modest results obtained for resveratrol in human clinical trials may be due, in part, to unknown mechanisms of action that prevent a more targeted or precision medicine approach. Studies in this laboratory have identified NR4A1 as a pro-oncogenic factor in solid tumor-derived cells and animal models [159]. NR4A1 also regulates cancer cell growth, survival, and migration/invasion, and this includes inactivation of AMPK. Activation of mTOR and these responses can be reversed by bis-indole derived (CDIM) NR4A1 antagonists. Many of the effects of CDIMs are similar to those caused by resveratrol in cancer.

In this study, we used H460 and H1299 lung cancer cells as models, and treatment with resveratrol decreased lung cancer cell growth, enhanced apoptosis, and decreased migration and invasion (Figs. 2-2 and 2-3). These results have previously been observed in lung cancer cells treated with resveratrol [270, 275], and our studies also show that comparable effects have been observed in H460 and H1299 cells after NR4A1 knockdown (Figs. 2-2 and 2-3) and after treatment with CDIM/NR4A1 antagonists [280]. These data suggested that resveratrol may be an NR4A1 ligand, and this was confirmed in direct binding and ITC assays, where the KD value for binding was in the

low mM range (Fig. 2-1). Docking resveratrol to the NR4A1 LBD (Fig. 2-1C) utilizing the Schrodinger Maestro modeling approach resulted in several favorable interactions (yellow dotted line) between resveratrol and specific amino acid residues of NR4A1 LBD [Ser110, Gul114, Arg184, Thr236 (aromatic, Leu239, and Ile260)]. Two unfavorable interactions (orange dotted line) between resveratrol and the NR4A1 LBD were also predicted (Leu239 and Ile260). Docking studies with the newly developed high affinity CDIM ligand bis(30-indolyl)- 1-(3,5-dichlorophenyl)methane (DIM-3,5-Cl2) [274] to the NR4A1 LBD (Fig. 2-1D) resulted in similar favorable interactions (yellow dotted line), as predicted for resveratrol, including specific interactions with Ser110, Glu114 (halogen bond), Arg184, Arg232 (aromatic), and Thr236 side chains, but also some differences. These differences in the interactions of resveratrol and DIM-3,5-Cl2 with amino acids in the ligand binding domain of NR4A1 are consistent with designation of these compounds as selective receptor modulators. The binding results coupled with the inhibitory effects of resveratrol on NR4A1-dependent transactivation (Fig. 2-1) demonstrate for the first time that resveratrol is an NR4A1 ligand that acts as a receptor antagonist and inhibits NR4A1-dependent transactivation in lung cancer cells.

We also examined a number of gene products previously shown to be regulated by CDIM/NR4A1 antagonists in cancer cells [159], and these include decreased expression of TXNDC5, IDH1, mTOR, and β1-integrin and induction of apoptosis gene products and activation of p-AMPK (Figs. 2-4, 2-5, and 2-6). Responses observed for resveratrol and NR4A1 knockdown were comparable, and β1-integrin was regulated by NR4A1

through interactions of NR4A1 as a cofactor of Sp1 bound to the GC-rich sites of the β1-integrin gene. Since knockdown of Sp4 in H1299 and H460 cells also decreases Sp1 expression, it was not possible to determine unequivocally a role for Sp4 in regulating β1-integrin gene expression via NR4A1/Sp4. The ChIP assay shows that both Sp1 and Sp4 bind the GC-rich integrin promoter, and it is possible that NR4A1 may coactivate both Sp1 and Sp4, and this process is blocked by resveratrol, as previously observed for CDIM/NR4A1 ligands [83, 162, 185, 281]. Thus, like CDIM/NR4A1 antagonists, resveratrol also inactivates NR4A1/Sp-regulated genes such as β1-integrin, and this further confirms that the mechanisms and functions of resveratrol are due, in part, to its activity as a NR4A1 antagonist.

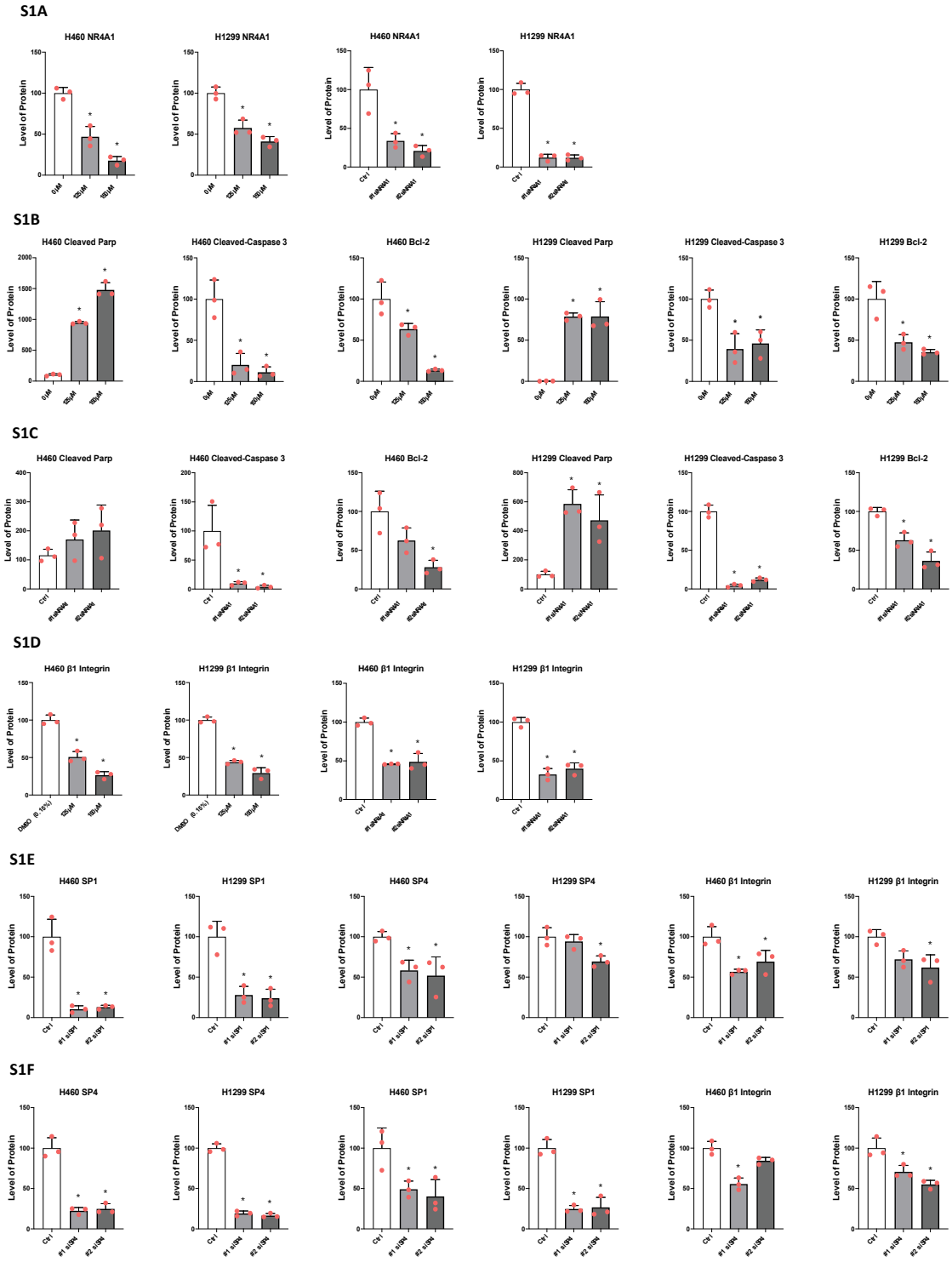
This study demonstrates for the first time that resveratrol binds with high affinity to NR4A1 and acts as an NR4A1 antagonist in lung cancer cell lines. Although the KD value for resveratrol is in the low μM range, indicating strong ligand-receptor interactions, the dose-response functional effects of resveratrol are in the 100–200 μM range in lung cancer cells [270, 275], and this is several orders of magnitude higher than the KD value. This difference may be due to several factors, including the effectiveness of the bound receptor complex to interact with nuclear cofactors, cellular uptake of resveratrol, and rapid metabolism (conjugation), which is commonly observed for other polyphenolics. Like many solid tumors, NR4A1 is overexpressed in many solid tumors and is a negative prognostic factor for patient survival [280]. This suggests that clinical applications of resveratrol in lung cancer chemotherapy may be more effective in

treating patients with tumors that overexpress NR4A1. It should also be noted that there is a long list of potential targets of resveratrol that include kinases, cytokines, cell signaling molecules, key genes involved in cancer cell proliferation, survival, and migration/invasion [240-250, 259-262]. This list also includes interactions with other receptors including nuclear receptor superfamily members. This study highlights the contribution of resveratrol as an NR4A1 ligand (antagonist) in lung cancer cells, and the effectiveness and contributions of this response to the overall anticancer activity of resveratrol may also be tumor-type specific and needs to be further investigated.

Supplemental Table 2-1.

Name of antibody	Vendor	Catlog #	Western blotting (Dilution)	ChIP (Mass)
β-actin	Sigma	A5316	3000	
NR4A1	Abcam	109180	1000	
c-Parp	Cell Signaling	9541	1000	
c-Caspase 3	Invitrogen	PA5-114687	500	
Bcl-2	Cell Signaling	4223	500	
TXNDC5	GeneTex	GTX106914	1000	
AMPK-α	Cell Signaling	5831	500	
p-AMPK-α	Cell Signaling	2535	500	
IDH1	Cell Signaling	8137	500	
m-TOR	Cell Signaling	2972	500	
p-m-TOR	Abcam	109268	500	
S6RP	Cell Signaling	2217	500	
p-S6RP	Cell Signaling	4858	1000	
4E-BP1	Cell Signaling	9644	1000	
p-4E-BP1	Cell Signaling	9451	1000	
β1-Integrin	Cell Signaling	9699	1000	
Sp1	Abcam	13370	1000	3μg
Sp4	Santa Cruz Biotechnology	SC-309124	500	3μg
pol II	Santa Cruz Biotechnology	47701		3μg
IgG	Cell Signaling	2729		3μg

Supplemental Figure 2-1.



CHAPTER III

PIPERLONGUMINE IS A LIGAND FOR THE ORPHAN NUCLEAR RECEPTOR 4A1 (NR4A1)

3.1. Introduction

Reactive oxygen species (ROS) and oxidative stress in both non-cancer and cancer cells is important for maintaining cellular homeostasis and inducing cell death [251-254]. ROS can play a beneficial role in both cancer and non-cancer cells however, drugs that induce ROS are being developed for cancer chemotherapy [253, 254]. The cytotoxicity of ROS inducers is associated with their induction of ROS which exceeds the redox buffering capacity of the cell. Some of the commonly used anticancer agents that induce ROS include arsenic trioxide, ionizing radiation, several anthracyclines such as doxorubicin, paclitaxel, and celecoxib [289-294]. For many, of the ROS-inducing anticancer agents their induction of ROS is a contributing factor to their anticancer activity, but it may not be the only factor. For example, doxorubicin not only induces ROS but also intercalates with DNA to inhibit synthesis and also inhibits progression of topoisomerase II [294]. A number of natural products and their synthetic analogs being developed as anticancer agents also induce ROS in cancer cells and in vivo tumor models. These compounds include isothiocyanates, curcumin, betulinic acid, piperlongumine, synthetic triterpenoids derived from oleanolic acid and glycyrrhetinic acid, celastrol and many others [295-302]. The mechanisms associated with drug-induced ROS are extensive and may be due to targeting mitochondria, inhibition of intracellular pathways/genes associated with redox, depletion of glutathione and other

intracellular reductants [253, 254, 303-305]. ROS activates or inactivates genes and pathways that lead to decreased cell growth, induction of apoptosis, inhibition of cell migration, invasion, and metastasis. For example, O'Hagan and coworkers initially reported that treatment of SW480 colon cancer cells with hydrogen peroxide, one form of ROS, induced rapid relocation of polycomb members, SIRT1 and DNA methyl transferences from non-GC-rich to GC-rich promoter sequences [306]. The resulting modulation of gene expression included downregulation of cMyc, and subsequent studies show that this can lead to decreased cell growth, survival and migration/invasion [295]. Studies in this laboratory have identified a series of bis-indole derived (CDIM) compounds that bind to the orphan nuclear receptor 4A1 (NR4A1) and act as inverse agonists to inhibit multiple pro-oncogenic genes/pathways associated with cancer cell growth, survival and migration/invasion [157, 159]. In some cell lines the CDIM compounds downregulate the pro-reductant thioredoxin domain containing (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) genes and this is accompanied by induction of ROS [160, 185, 210, 279, 307]. Moreover, knockdown of NR4A1 or TXNDC5 by RNA interference also induced ROS in some cancer cell lines suggesting that some ROS-inducing anticancer agents may also be NR4A1 ligands and that some of their anticancer activities may due, in part, to their activity as inverse NR4A1 agonists in cancer cells. This is consistent with results of studies with the potent ROS-inducing anticancer agent celastrol [299] which has now also been identified as an NR4A1 ligand [171]. This observation is relevant with respect to drug development since NR4A1 has been implicated as a potential drug target for multiple diseases including cancer and many

other inflammatory diseases [273, 308-310]. In this study we have investigated the anticancer activity of the well-known ROS-inducing anticancer agent piperlongumine [300, 301] and show for the first time that this compound is an NR4A1 ligand acting as an inverse agonist in colon cancer cells.

3.2. Materials and Methods

3.2.1. Cell culture, reagents, and antibodies

RKO, SW480 and HCT116 (RRID: CVCL_0291) colon cancer cells are purchased from American Type Culture Collection (Manassas, VA) and validation on selected cell lines were determined by biosynthesis (Lewisville, TX). Cells are cultured in DMEM medium with 10% FBS at 37 °C in the presence of 5% CO2. The details of antibody using for Western Blotting and ChIP assays are shown in Supplemental Table 1. 1,1-Bis(3'-indolyl)-1-(3,5 dichlorophenyl)methane (DIM-3,5-Cl2) was synthesized by coupling indole and 3,5 dichlorobenzaldehyde as described [311].

3.2.2. Direct binding assay

The recombinant LBD of NR4A1 (0.5 μmol/L) in 1.0 ml of phosphate buffered saline (pH 7.4) was incubated for 3 minutes at 25 °C in a temperature-controlled fluorescence spectrometer (Varian Cary Eclipse). Fluorescence was measured using an excitation wavelength of 285 nm (excitation slit width = 5nm) and an emission wavelength ranging from 300-420 nm (emission slit width = 5 nm). Aliquots of piperlongumine in DMSO

were added, incubated at 25 °C for 3 minutes. The loss of fluorescence curve was measured, and K_D values were determined from the net fluorescence curve (background ligand fluorescence is subtracted).

3.2.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was used to determine the ligand binding constant (KD) to NR4A1 utilizing an Affinity ITC (TA Instruments, New Castle, DE). Briefly, the experimental setup was as follows. The ITC sample cell contained 250 μL of NR4A1 protein (ligand binding domain, LBD) at a concentration of 20 μmol/L in buffer containing 20 mmol sodium phosphate/L (pH 7.4), 5% glycerol, and 1.0% ethanol. The ligand titrant was prepared in the same buffer as above at a ligand concentration of 100 μmol/L. The initial ligand stock solution was prepared at a final concentration of 20 mmol ligand/L DMSO prior to preparation of the ligand titrant. The ligand titration into protein was performed at 25 °C with a stir rate of 125 rpm. Each ligand injection volume was 2.5 μL followed by up to 300 sec to measure the total heat flow required to maintain constant temperature. A total of thirty injections were done for each ligand/NR4A1 combination. In a separate set of injections, the same ligand was injected into buffer only (no protein) in order to determine heat flow as a result of ligand dilution into buffer. The ligand/buffer values were subtracted from the ligand/protein values prior to data analysis using the Affinity ITC manufacturer-supplied data analysis software package. The resulting data are plotted as heat flow (μJ) versus the molar ratio of injected ligand to NR4A1 in the sample cell.

3.2.4. Computation-based molecular modeling

Molecular modeling studies were conducted using Maestro (Schrödinger Release 2020-1, Schrödinger, LLC, New York, NY, 2020). The version of Maestro used for these studies is licensed to the Laboratory for Molecular Simulation (LMS), a Texas A&M University core user facility for molecular modeling and is associated with the Texas A&M University High Performance Research Computing (HPRC) facility (College Station, TX, 77843). All Maestro-associated applications were accessed via the graphical user interface (GUI) VNC interactive application through the HPRC Ada OnDemand portal. The crystal structure coordinates for human orphan nuclear receptor NR4A1 ligand binding domain (LBD) (36) were downloaded from the Protein Data Bank (https://www.rcsb.org; PDB ID 3V3Q). The human NR4A1 LBD crystal structure was prepared for ligand docking utilizing the Maestro Protein Preparation Wizard; restrained minimization of the protein structure was performed utilizing the OPLS3e force field. Each ligand (piperlongumine or DIM-3,5-Cl2) three-dimensional structure was prepared for docking utilizing the Maestro LigPrep, again using the OPLS3e force field. Maestro Glide (37-39) was utilized with the default settings to dock each prepared ligand to the prepared protein, predict the lowest energy ligand binding orientation, and calculate the predicted binding energy in units of kcal/mol.

3.2.5. Cell proliferation assay

Cell proliferation was investigated using XTT Cell Viability Kit (Cell Signaling Biotechnology) according to the manufacturer's instructions. Cells (1.5×10^4/well) were

plated in 100 μl of plating medium (as above) in 96-well plates and allowed to attach for 24 hours. The medium was then changed to DMEM containing 2.5% charcoal-stripped FBS, and either vehicle (dimethyl sulfoxide (DMSO)) or designed concentrations of compounds in DMSO were added. After 24 and 48 hours of culture, 35 μL of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl) 3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the each well. The optical density was read at 450 nm wavelength in a plate reader after 4 hours of incubation. All determinations were replicated in at least three separate experiments.

3.2.6. Transfection and luciferase assay

Cells were plated on 12-well plates at 5×10^4/well in DMEM medium supplemented with 2.5% charcoal-stripped FBS. After 24 hours growth, various amounts of plasmid DNA [i.e., UASx5-Luc (400 ng), GAL4-NR4A1 (250 ng) and β-gal (250 ng)] were cotransfected into each well by GeneJuice Transfection reagent (Millipore Sigma, Darmstadt, Germany) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with plating media (as indicated above) containing either solvent (DMSO) or the indicated concentration of compound (in DMSO) for 18 hours. Cells were then lysed using a freeze–thaw protocol and 30 μL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against

corresponding β- gal activity values as well as protein concentrations determined by Bradford assay.

3.2.7. Annexin V staining assay

Annexin V staining assay was performed using Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (Invitrogen, Carlsbad, CA). Briefly, cells were seeded in 6 well plates followed by various drug treatments. The cells were then washed with ice cold PBS and 5 μL Alexa Fluor® 488 Annexin V with 100 μg/mL PI (as per the manufacturer instructions) were added to the cells and incubated for 15 minutes and the cells were determined by Accuri flow cytometer.

3.2.8. Boyden chamber invasion assay and scratch migration assay

Attached cells (2.0×10^5) were treated with DMSO or with different concentration of piperlongumine in DMEM medium supplemented with 2.5% charcoal stripped FBS for 24 hours. For Boyden chamber invasion assay, 1.0×10^5 cells from each treatment condition were allowed to invade through the Boyden Chamber for 48 hours. Cells that invaded into the Boyden Chamber were fixed using formaldehyde, stained, and then counted. For the scratch migration assay, cells were grown to 90% confluency in 6-well plates then scratched with a 200 μL sterile pipette tip and washed with PBS to remove detached cells from the plates. Cells were maintained in an incubator with DMSO or indicated treatments for 48 hours and cells were then fixed with 4% formaldehyde and

stained with crystal violate solution. The wound gap was observed under AMG EVOS fl microscope. At least 3 replicates were performed for each treatment group.

3.2.9. Western blot analysis

Cells (3.0×10^5) were seeded on 6-well plate and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer RIPA (Thermo Scientific, Waltham, MA) that contained protease and phosphatase inhibitors (GenDEPOT, Baker, TX). The total protein in the lysates was quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel; 35 μg of whole cell lysate were run in 12% of SDS page gels for survivin and c-caspase-3 proteins; 35 μg of whole cell lysate were run in 8% of SDS page gels for G9a, mTOR and p-mTOR proteins and 25 μg of whole cell lysate were run in 10% of SDS page gels for the remaining proteins. The proteins from the gel were transferred to a PVDF membrane, then blocked for one hour using 5% skimmed milk. The membranes were incubated with primary antibody for 12 hours at 4 °C, then washed with Tris-buffered saline and Polysorbate 20 (TBST) and incubated with HRP-linked secondary antibody for 1 hour at 20 °C. The membranes were further washed with TBST and treated with Immobilon western chemiluminescence HRP-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA).

3.2.10. ChIP assay

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Cells (3×10^7) were treated with DMSO or indicated concertation of piperlongumine for 24 hours. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with 3 μg of normal IgG (abcam), NR4A1 (Abcam), Sp1 (Abcam), or RNA polymerase II (pol II; Abcam) antibodies and protein G-conjugated magnetic beads at 4 °C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. Reversed cross-link DNA was prepared by proteinase K digestion followed by Chromatin IP DNA purification (Active Motif). Then purified DNA products were analyzed by quantitative real-time PCR using amfiSure qGreen Q-PCR master mix (genDEPOT) using the manufacturer's protocol. The primers for detection of the G9a promoter region were F: 5-CAGATGGGGACAGAGACGC-3, R: 5-CCCGGAGCA TTGCACG-3.

3.2.11. Statistical analysis

Each assay was performed in triplicate and the results were presented as means with standard deviation (SD). The statistical significance of differences between the treatment

groups was determined by Dunnett's multiple comparison test in ordinary one-way ANOVA. Gel analysis of Western Blotting was done using ImageJ (1.53K) software (RRID:SCR_003070). GraphPad Prism 8 (Version 8.4.3) software (RRID:SCR_002798) was used for analysis of variance and determine statistical significance. Data with a P value of less than 0.05 were considered statistically significant and indicated (*) in the figures.

3.3. Results

Based on the functional similarities between ROS-inducing anticancer agents and NR4A1 inverse agonists in cancer cell lines we investigated the potential activity of piperlongumine as an NR4A1 ligand. Figure 3-1A illustrates the direct binding of piperlongumine to the ligand binding domain (LBD) of NR4A1 using an assay which measures the loss of fluorescence of a Trp residue in the LBD [210]. The results show that piperlongumine binds NR4A1 with a K_D value of 7.1 μM and as a positive control for this assay we observed a similar binding curve for the known NR4A1 ligand celastrol with a K_D value of 2.3 μM (data not shown). Figure 3-1B illustrates the interaction of piperlongumine and NR4A1 as determined in the ITC assay and the K_D and ΔG values were 4.97 μmol/L and -30.9 kJ/mol respectively. The lower K_D value in the ITC assay may represent interactions of piperlongumine not only within the binding pocket but also other sites in the LBD or NR4A1. Figure 3-1C illustrates Maestro/Schrodinger modeling of piperlongumine with the LBD of NR4A1 and shows interactions of piperlongumine with hydrophobic (Pro266, Thr264, Phe261, Leu108, Glu109, Ser110), polar (Ala111,

Phe112, Leu113, Glu114, Leu239), and negatively charged (Thr236, Cys235, Arg232, Val179 and Arg184) amino acids. These results (Fig. 1A-1C) clearly demonstrate that piperlongumine is an NR4A1 ligand and this is further confirmed in NR4A1-dependent transactivation studies in cells transfected with a GAL4-NR4A1 chimera and a GAL4-dependent reporter gene (UAS-luc). The results show that piperlongumine decreased luciferase activity in RKO (Fig. 3-1D), SW480 (Fig. 3-1E), and HCT116 (Fig. 3-1F) colon cancer cells demonstrating the NR4A1 inverse agonist activity of piperlongumine for this transactivation response.

In cancer cells inverse NR4A1 agonists decrease cancer cell growth, induce apoptosis and inhibit migration/invasion [159, 210] and treatment of RKO, SW480 and HCT116 cells with 7.5 and 15 μM piperlongumine inhibited growth (Figure 3-2A to 3-2C) as determined in an XTT assay. Treatment with 7.5 and 15 μM piperlongumine caused the morphology of colon cancer cells to visibly shrink and changed to a rounded shape (Fig. 3-2D). Moreover, this same treatment protocol also induced Annexin V and PI staining in the colon cancer cell lines (Fig. 3-2E and 3-2F), and this is a marker of drug-induced apoptosis and necrosis.

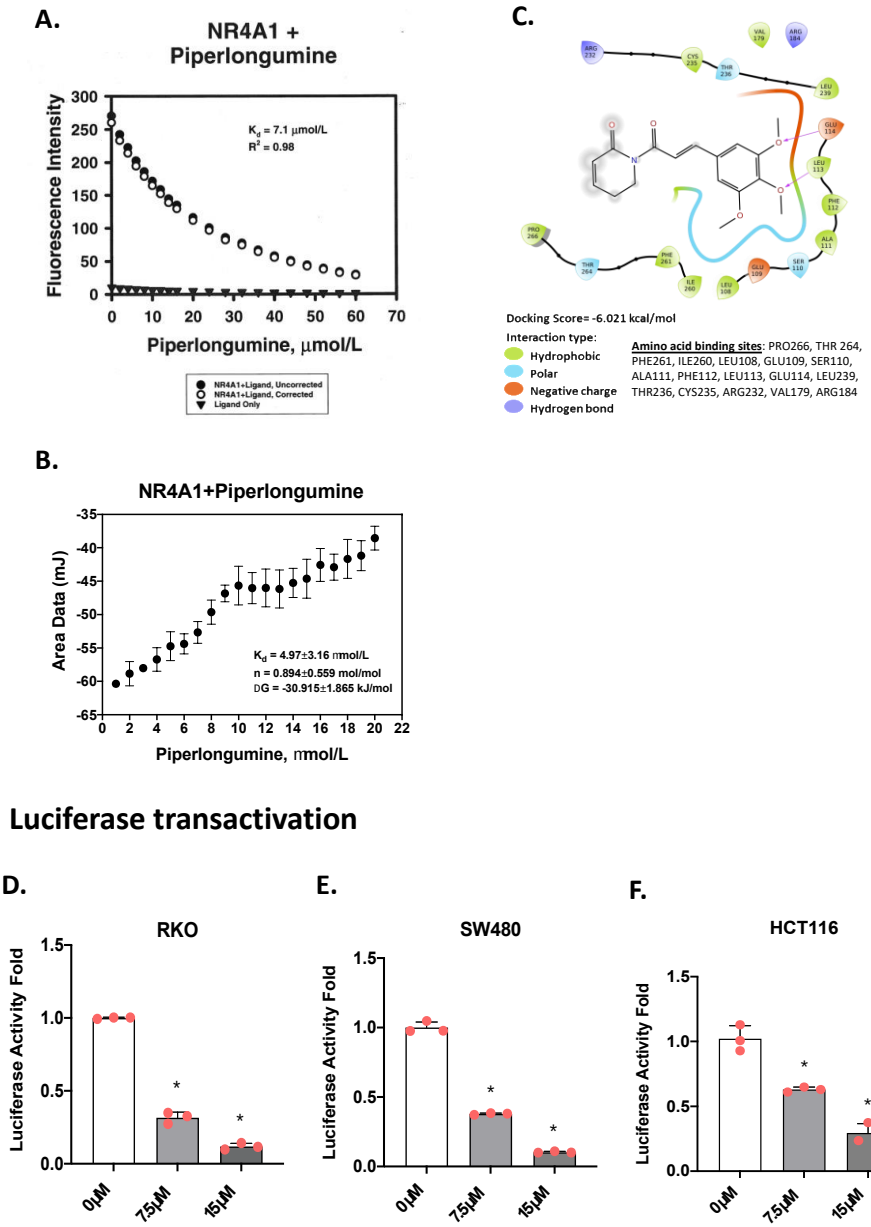


Figure 3-1. Piperlongumine as an NR4A1 ligand. A) Direct binding of piperlongumine to the LBD of NR4A1 by measuring loss of Trp fluorescence as outlined in the Methods; B) Binding of piperlongumine to the LBD of NR4A1 was also determined using an ITC assay as outlined in the Methods; C) Piperlongumine interactions with LBD of NR4A1 were modeled using Schrodinger/Maestro as outlined in the Methods. Effects of piperlongumine on NR4A1-dependent transactivation was determined in RKO (D), SW480 (E) and HCT116 (F) colon cancer cells transfected with a GAL4-NR4A1 chimera and a UAS-luc reporter gene as outlined in the Methods. Results (D-F) are expressed as means ± SD for at least 3 determinations and significant ($p < 0.05$) effects of piperlongumine compared to control (DMSO) are indicated (*).

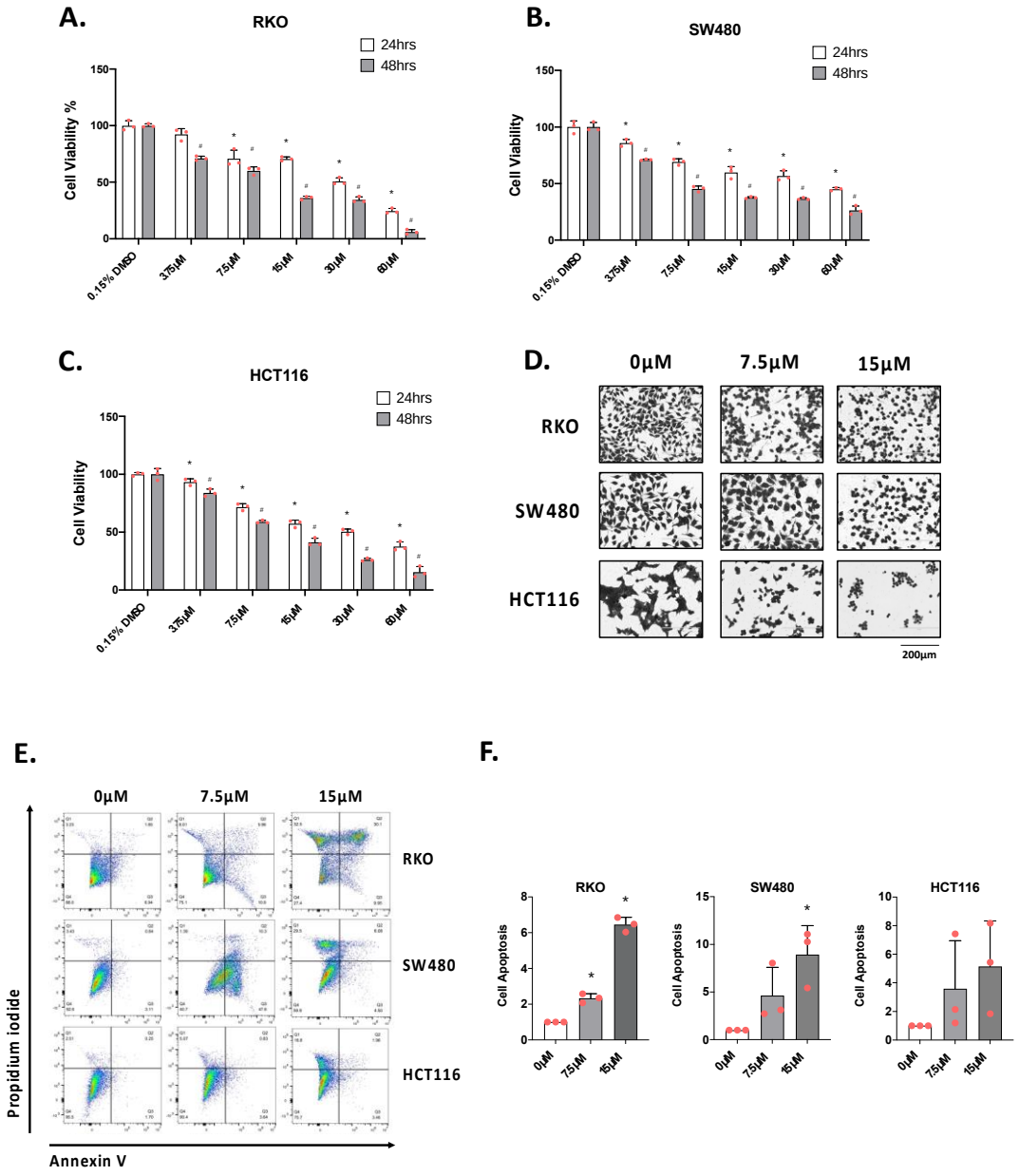


Figure 3-2. Piperlongumine inhibits colon cancer cell growth and induces Annexin V staining. RKO (A), SW480 (B) and HCT116 (C) cell were treated with different concentrations of piperlongumine for 24 or 48 hours and effect were measured using XTT assay as outlined in the Methods. D) Colon cancer cells were treated for 24 hours with 7.5 or 15 μM piperlongumine and changes in cell morphology were determined as outlined in the Methods. Colon cancer cells were treated for 24 hours with piperlongumine and changes in Annexin V were determined by FACS analysis (E) and quantitated (F) as outlined in the Methods. Results for Annexin V staining are expressed as means ± SD for at least 3 determinations and significant ($p < 0.05$) induction is indicated (*).

Figure 3 illustrates that after treatment with piperlongumine for 24 hours there was a decrease in the growth promoting oncogene cMyc and several markers of apoptosis, namely decreased bcl-2 and survivin and increased cleaved caspase 3 and PARP in the 3 colon cancer cell lines (Fig. 3-3A to 3-3C). These results coupled with piperlongumine-dependent inhibition of colon cancer cell migration and invasion in a scratch and Boyden chamber assay (Figures 3-3D to 3-3E) demonstrate that piperlongumine affects functional responses in colon cancer cells that are consistent with their activity as inverse NR4A1 agonists and effects of NR4A1 knockdown [159]. Quantitation of the results illustrated in Figure 3-3 are summarized in Supplemental Figure 3-1.

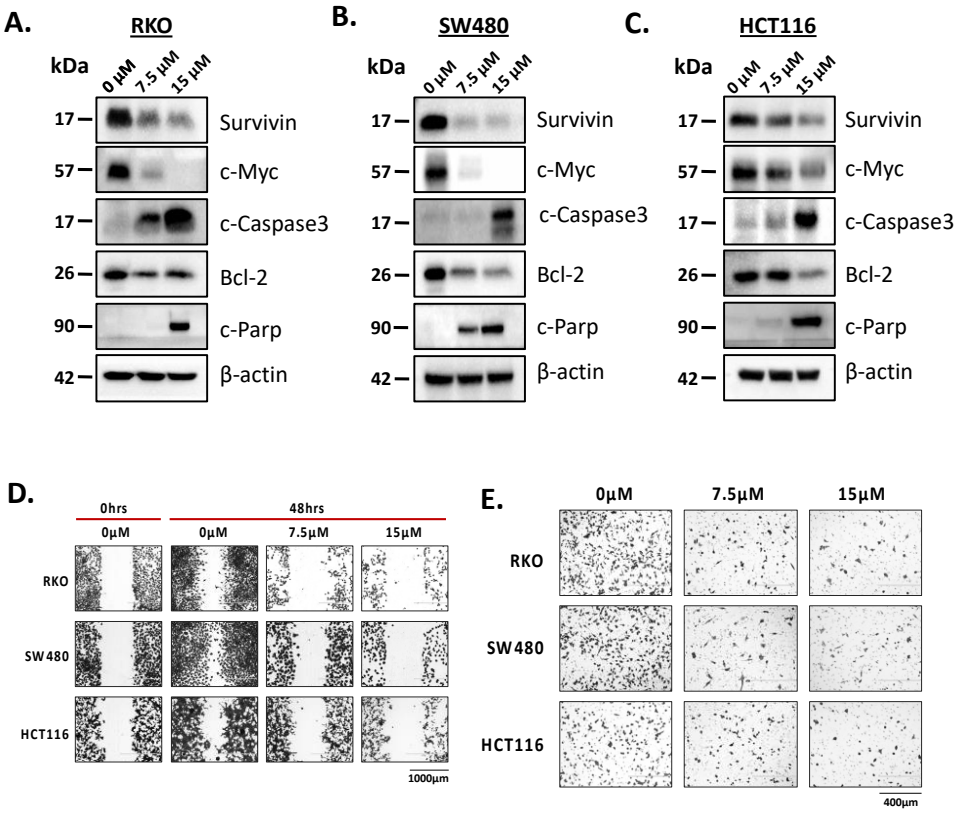


Figure 3-3. Piperlongumine induces apoptosis, inhibits migration and invasion in colon cancer cell lines. RKO (A), SW480 (B) and HCT116 (C) colon cancer cells were treated with 7.5 or 15 µM piperlongumine for 24 hours and whole cell lysates were obtained and analyzed by western blots as outlined in the Methods and bands were quantitated relative to β-actin and intensities are given as means ± SD for at least 3 determinations and significant effects ($p < 0.05$) relative to the control (DMSO) treatment groups are indicated (*). Colon cancer cells were treated with piperlongumine for 24 hours and effects on cell migration and cell invasion (D, E) were determined in scratch and Boyden chamber assays respectively as outlined in the Methods. Quantitative results are expressed as means ± SD for at least 3 determinations and significant ($p < 0.05$) differences between control (DMSO) and piperlongumine-treated cells is indicated (*).

One of the hallmarks of NR4A1 inverse agonists is the downregulation of pro-reductant genes such as TXNDC5 and IDH-1 which results in increased reactive oxygen species and induction of the oxygen sensor sestrin2 (SESN2) and SESN 2-dependent activation of AMPK [160, 185, 210, 279, 283, 307]. Treatment of the colon cancer cells with piperlongumine resulted in downregulation of IDH1 in RKO (Figure 3-4A), SW480 (Fig. 3-4B), and HCT116 (Not significant) (Fig. 3-4C) cells and selective

downregulation of TXNDC5 in only SW480 cells. Induction of sestrin2 and activation (increased phosphorylation) of AMPK was observed in all 3 cell lines. NR4A1 regulates multiple reductant genes [279] and their selective regulation by bis-indole-derived CDIMs and piperlongumine is consistent with their activity as selective NR4A1 modulators.

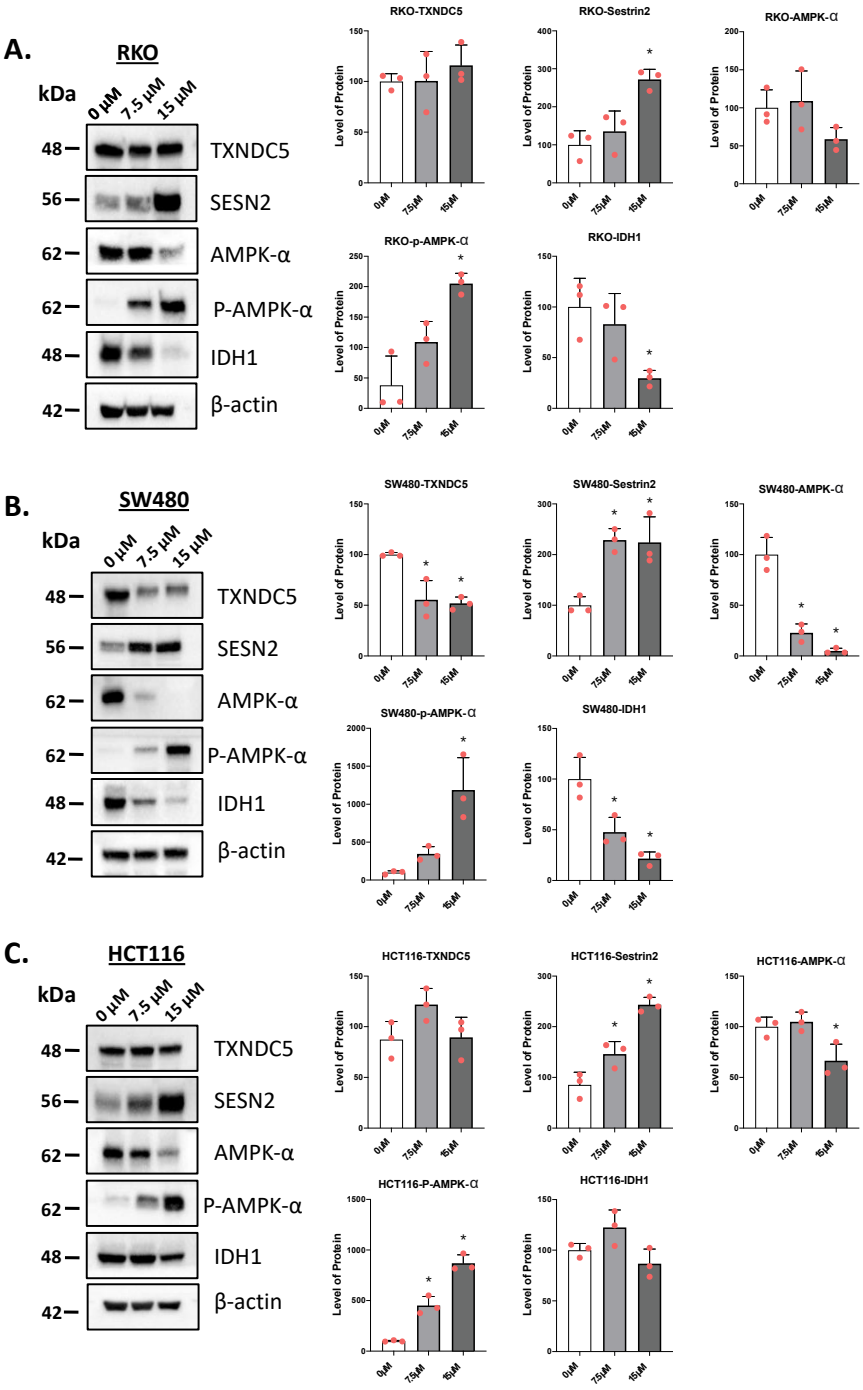


Figure 3-4. Piperlongumine affects redox in colon cancer cell lines. RKO (A), SW480 (B) and HCT116 (C) colon cancer cells were treated with 7.5 or 15 µM piperlongumine for 24 hours and whole cell lysates were obtained and analyzed by western blots as outlined in the Methods and bands were quantitated relative to β-actin. Band intensities are given as means ± SD for at least 3 determinations and significant effects ($p < 0.05$) relative to the control (DMSO) treatment groups are indicated (*).

Results illustrated in Figure 3-5 summarize effects of piperlongumine alone on induction of ROS and sestrin2 and effects of piperlongumine in combination with the antioxidant glutathione. Treatment of colon cancer cells with piperlongumine increases ROS-dependent fluorescence in all 3 cell which is clue to oxidation of the cell permeant DCFDA into its fluorescent metabolite (Figures 3-5A to 3-5C). In addition, this was accompanied by induction sestrin2 (Figs 3-5D to 3-5F) and the magnitude of the fluorescent and sestrin2 induction responses were decreased after cotreatment with glutathione. Sestrin2-dependent activation of AMPKα inhibits mTOR signaling and results in Figures 3-6A to 3-6C show that piperlongumine significantly decreased levels of phosphor-mTOR in the 3 colon cancer cell lines. Moreover, inhibition of mTOR is accompanied by significantly decreased phosphorylation of 4E-BP1 and p70S6 in the 3 colon cancer cell lines. Previous studies have demonstrated that the histone methyltransferase G9a gene is regulated by NR4A1/Sp1 where NR4A1 acts as a ligand-dependent cofactor of Sp1 which in turn is bound to the GC-rich promoter of the G9a gene [283]. Results in Figures 3-7A to 3-7C show that piperlongumine downregulates G9a expression in RKO, SW480 and HCT116 cells and this parallels effects observed for bis-indole derived CDIM compound, quercetin and kaempferol which also bind NR4A1 and act as inverse NR4A1 agonists [283, 312]. Chromatin immunoprecipitation and QPCR were used to quantitatively detect interactions of NR4A1 and Sp1 with the GC-rich G9a promoter (Fig. 3-7D) in SW480 cells. Treatment with piperlongumine had minimal effects on Sp1 binding but significantly decreased NR4A1 interactions in this region of the G9a promoter and similar results were previously observed using bis-

indole derived NR4A1 ligands in Rh30 cells [283]. This data confirms interaction of piperlongumine and NR4A1 and their effects on gene expression and are consistent with the activity of piperlongumine as an inverse NR4A1 agonist.

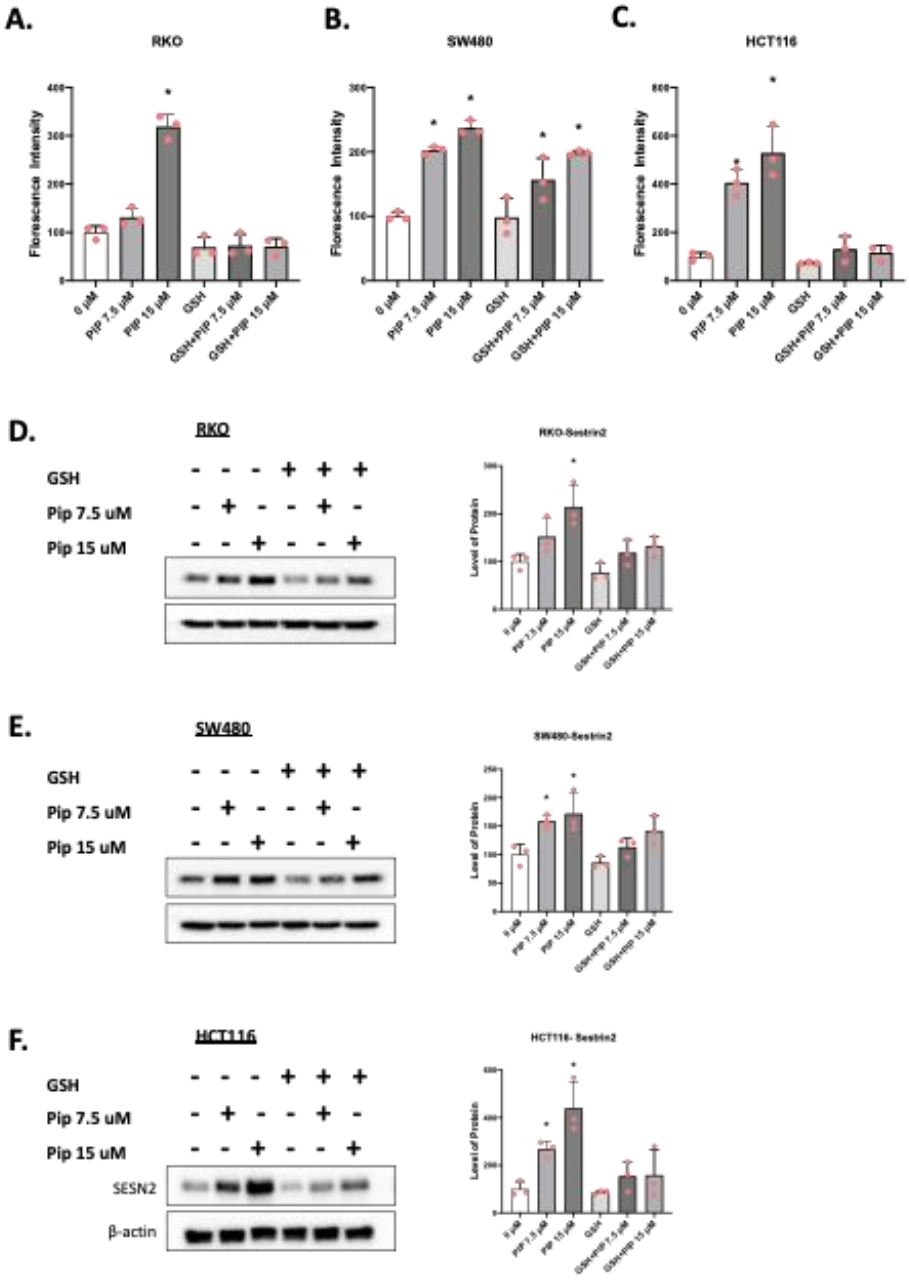


Figure 3 Error! No text of specified style in document.**-5. Piperlongumine induces ROS and sestrin2. Treatment of RKO (A), SW480 (B) and HCT116 (C) cells with piperlongumine, 5 μM glutathione and a combination of piperlongumine plus glutathione. Piperlongumine increased ROS induction due to metabolism of cell permeable DCFDA as outlined in the Methods. The same treatment protocol was used in RKO (D), SW480 (E) and HCT116 (F) cells and whole cell lysates were analyzed by western blots and band intensifies were quantitated relative to β-actin. Results are expressed as means ± SD for at least 3 determinations and significant effects ($p < 0.05$) compared to control are indicated (*) and attenuation by glutathione is also indicated (*).**

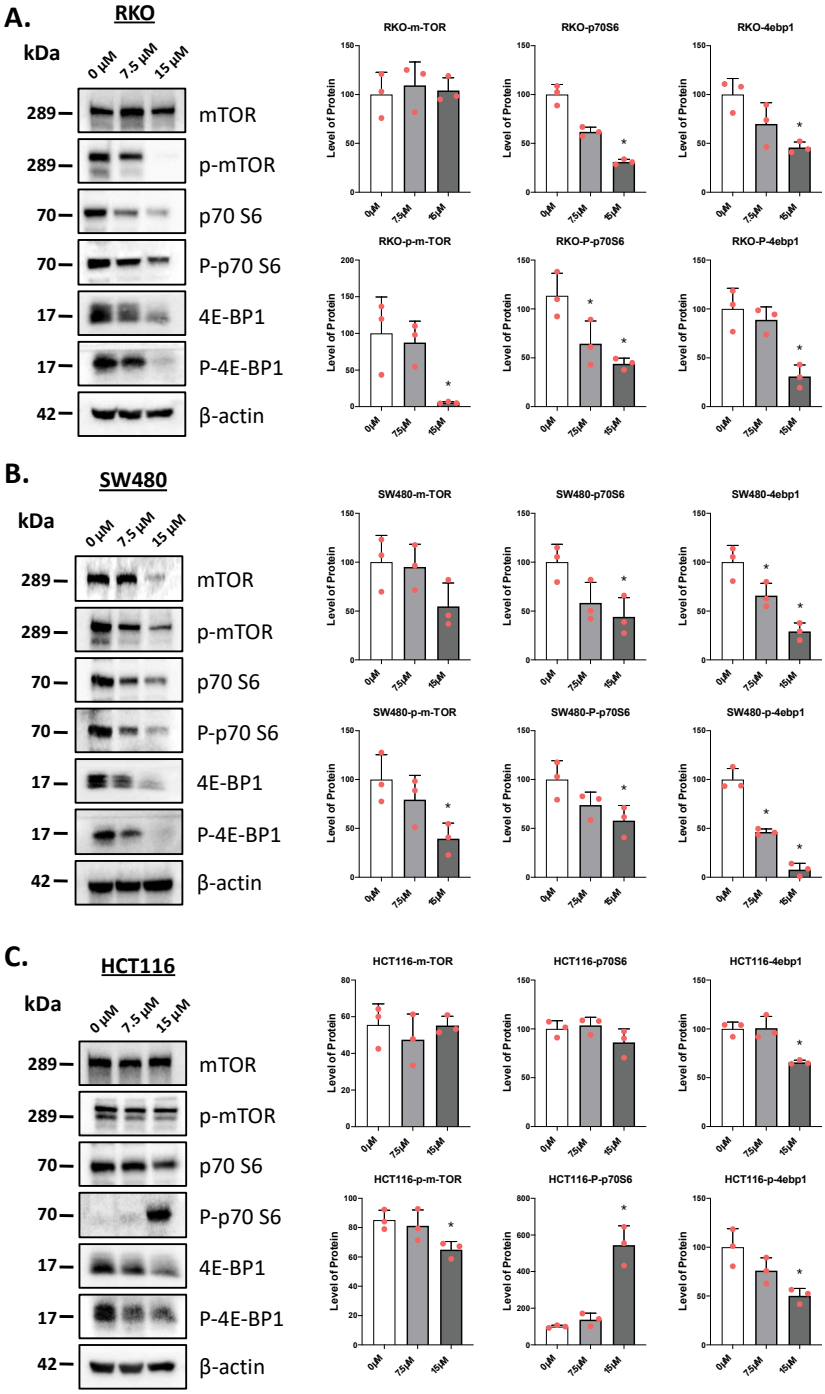


Figure 3-6. Piperlongumine inhibits mTOR in colon cancer cells. RKO (A), SW480 (B) and HCT116 (C) cells were treated with piperlongumine for 24 hours and whole cell lysates were obtained and analyzed by western blots and band intensities were quantitated relative to β-actin. Band intensities are given as means ± SD for at least 3 determination and significant changes ($p < 0.05$) compared to control (DMSO) are indicated (*).

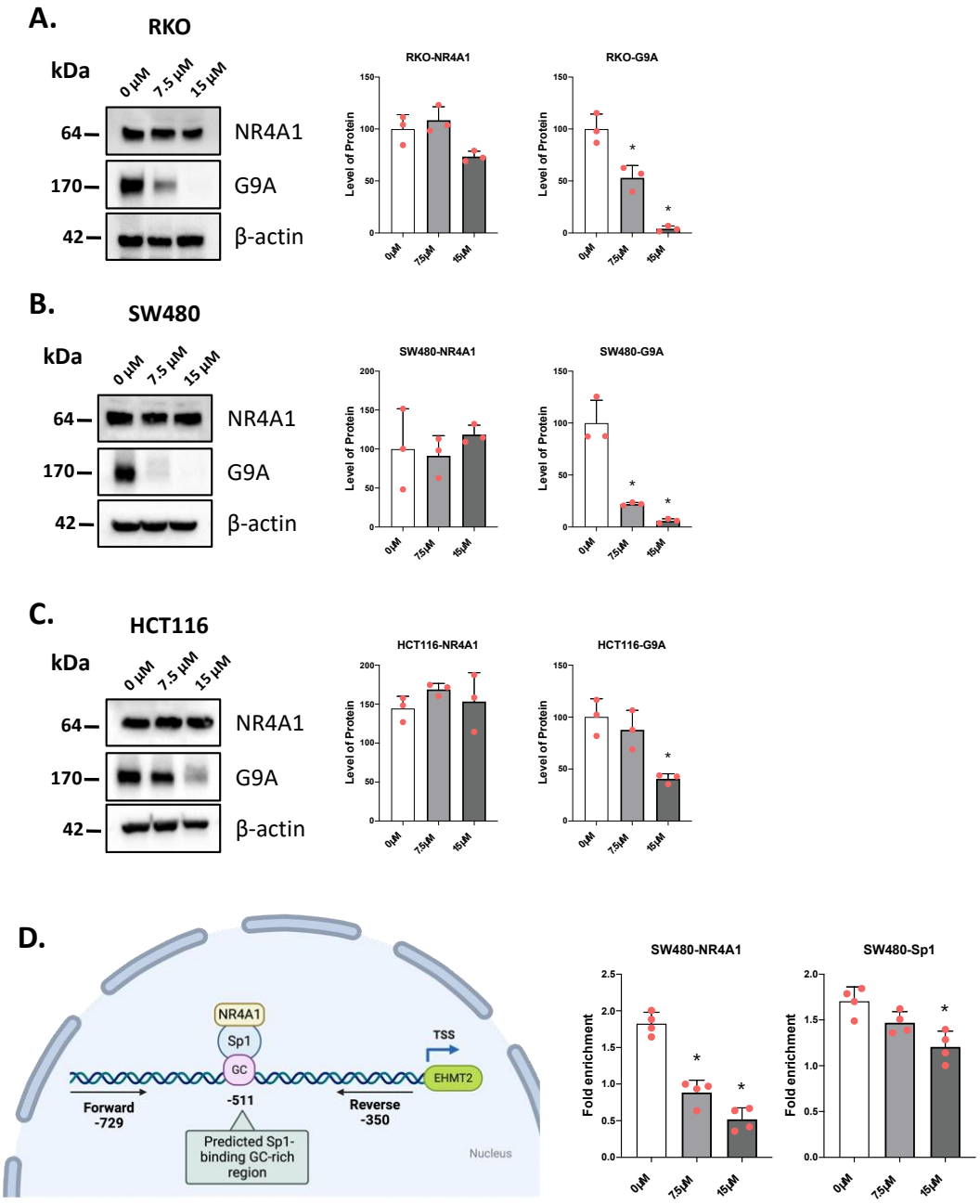


Figure 3-7. Mechanism of G9a regulation by piperlongumine. RKO (A), SW480 (B) and HCT116 (C) colon cancer cells were treated with 7.5 or 15 µM piperlongumine for 24 hours and whole cell lysates were obtained and analyzed by western blots as outlined in the Methods and bands were quantitated relative to β-actin. D) A ChIP assay was used to determine the interactions of NR4A1 and Sp1 with the GC-rich region of the G9a gene promoter as outlined in the Methods using primers that encompass the GC-rich region. QPCR was used to analyze fold enrichment of NR4A1 and Sp1 associated with the GC-rich promoter region and QPCR intensities are given as means ± SD for at least 3 determinations and significant effects ($p < 0.05$) relative to the control (DMSO) treatment groups are indicated (*).

3.4. Discussion

The orphan nuclear receptor NR4A1 is an immediate early gene that plays an important role in maintaining cellular homeostasis and in pathophysiology [273, 308-310]. For example, NR4A1 is elevated by stressors and inflammatory agents and levels are increased in many solid tumors, fibrosis, some cardiovascular, neuronal and metabolic diseases [273, 308-310]. NR4A1 ligands such as cytosporone B act as disease-specific agonists or inverse agonists for relieving symptoms of these diseases including cancer [153, 158]. Studies in this laboratory have characterized the inverse agonist activities of bis-indole derived NR4A1 ligands in solid tumor-derived cancers and their beneficial effects in neuronal disease, endometriosis, and glucose uptake into muscle cells has also been reported [157, 159, 160, 168, 185, 194, 210, 212, 274, 279, 307, 312-314]. Many therapeutic agents including some health-promoting natural products exhibit multiple activities and this can lead to drug repurposing which allows a particular drug to be used for more than one mechanism-based response [315]. Natural products including several anticancer agents induce responses similar to that observed for bis-indole derived NR4A1 ligands which act as inverse agonists and inhibit NR4A1-dependent pro-oncogenic genes and pathways [159]. For example, the flavonoid kaempferol downregulated expression of G9a in gastric cancer cells [316] and studies in this laboratory show the flavonoids quercetin and kaempferol bind NR4A1 and downregulate G9a and NR4A1-regulated genes in Rh30 cells [177] and similar results have now been observed for piperlongumine (Fig. 3-7). Several other natural products that act as anticancer agents including cytosporone B, celastrol, resveratrol and some alkaloids

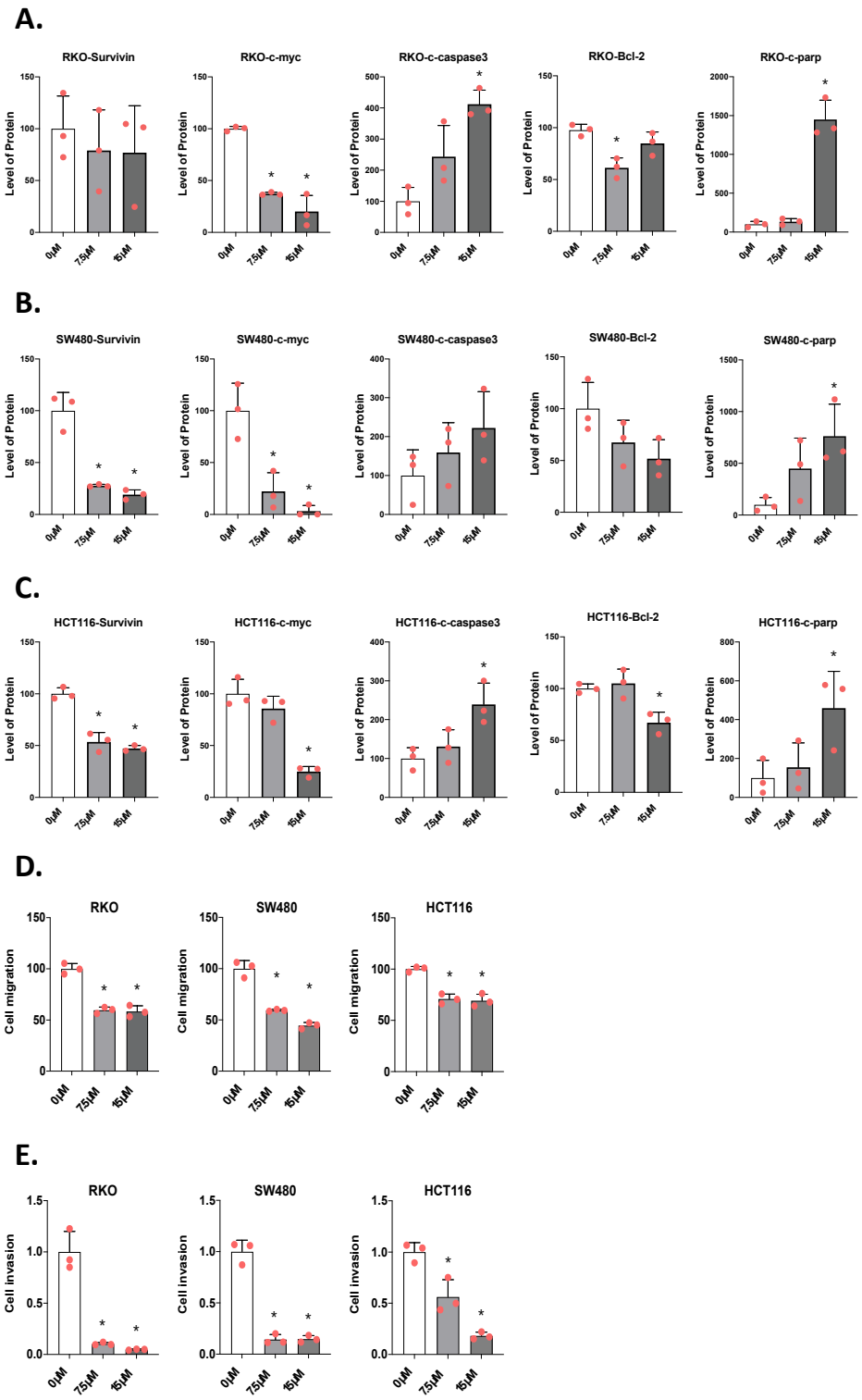
have also been identified as NR4A1 ligands and this will help facilitate their repurposing for treating diseases where NR4A1 is an important target [153, 157, 171, 177, 311]. In this study we show that piperlongumine binds NR4A1 and inhibits NR4A1-dependent transactivation in colon cancer cells. Like other NR4A1 inverse agonists, piperlongumine inhibits cell growth, induces apoptosis, inhibits migration and invasion, acts as an mTOR inhibitor induces ROS and modulates expression of several NR4A1-regulated genes [159]. Moreover, these responses are mimicked in several cancer cell lines after knockdown of NR4A1 [160, 185, 210, 279, 307]. It should also be noted that there were cell context dependent differences in the effects of piperlongumine with respect to induction of IDH-1 and TXNDC5 (Fig. 3-4). We also observed that piperlongumine did not affect expression of other genes such as β-1 and β3-integrins (data not shown) which are downregulated by bis-indole derived NR4A1 inverse agonists [159]. This selectivity of piperlongumine and other compounds that bind NR4A1 may be due to their activity as selective NR4A1 ligands which is observed for many other nuclear receptors [317]. The selectivity of receptor ligands is associated with multiple factors including their ligand structure-dependent induced conformational differences of the receptor and subsequent interactions with cell specific nuclear cofactors. In this study modeling of NR4A1-piperlongumine interactions showed ligand interactions with multiple amino acid side chains within the LBD (Fig. 3-1). Previous modeling studies showed that NR4A1 interactions with structurally diverse ligands showed both common and different amino acid side chains within the LBD. For example, the key amino acid side chain interactions were observed for the following

compounds; quercetin (Glu109, Phe112, Leu113, Glu114) [177], 1,1-bis(3'-indolyl)-1-(3,5-dichlorophenyl) methane (Ser110, Glu114, Arg184, Arg232 and Thr236) and resveratrol (Ser110, Leu113, Glu114, Arg184, Thr236, Leu239, and Ile-260) [311]. Thus, NR4A1 ligands show both common and different interactions with amino acid side-chains within the NR4A1 LBD and this may contribute to their activity as selective receptor modulators. In summary results of this study show that piperlongumine is an NR4A1 ligand that acts as an inverse agonist in colon cancer cells. These results indicate that the anticancer activity of piperlongumine is due in part, to its inactivation of NR4A1 and effects of this compound in other disease models where NR4A1 is a drug target are currently being investigated.

Supplemental Table 3-1.

Name of antibody	Vendor	Catlog #	Western blotting (Dilution)	ChIP (Mass)
β-actin	Sigma	A5316	3000	
NR4A1	Abcam	109180	1000	3μg
Survivin	R&D	AF886	500	
c-Myc	Abcam	Ab185656	500	
c-Parp	Cell Signaling	9541	1000	
c-Caspase 3	Invitrogen	PA5-114687	500	
Bcl-2	Cell Signaling	4223	500	
TXNDC5	GeneTex	GTX106914	1000	
Sestrin2	Abcam	18907	1000	
AMPK-α	Cell Signaling	5831	500	
p-AMPK-α	Cell Signaling	2535	500	
IDH1	Cell Signaling	8137	500	
m-TOR	Cell Signaling	2972	500	
p-m-TOR	Abcam	109268	500	
p70S6	Cell Signaling	9202	500	
P-p70S6	Cell Signaling	9205	500	
4E-BP1	Cell Signaling	9644	1000	
p-4E-BP1	Cell Signaling	9451	1000	
G9a	Cell Signaling	68851	1000	
Sp1	Abcam	13370	1000	3μg
pol II	Abcam	264350		3μg
IgG	Abcam	171870		3μg

Supplemental Figure 3-1.



CHAPTER IV

FLAVONOIDS QUERCETIN AND KAEMPFEROL ARE NR4A1 ANTAGONIST AND SUPPRESS ENDOMETRIOSIS

4.1. Introduction

Endometriosis is a common but complex estrogen-dependent inflammatory disease that occurs in cells lining the uterus and is implanted at distal sites such as peritoneal surfaces, bowel, and ovaries. Over 5,500,000 women in the United States and 176,000,000 women worldwide suffer from symptoms of endometriosis, pelvic pain, and infertility [318-321]. Once diagnosed, the staging of the disease (i.e., stage I – IV) and its location are essential for determining the appropriate treatment regimen. Excision surgery and hormonal therapy (such as progestins, oral contraceptives, and GnRH antagonists) to systemically deplete estrogen levels have been employed for treating endometriosis patients [322-328]. However, the current treatments for endometriosis do not effectively relieve endometriosis symptoms and causes adverse effects in other estrogen-target tissues, such as bone and brain, because hormonal therapy causes post-menopausal symptoms in endometriosis patients [320-322]. Therefore, the development of new non-hormonal therapies to improve the efficacy of endometriosis treatment without the accompanying adverse effects of current endometriosis treatment is highly desirable [320, 321]. Nuclear receptor 4A1 (NR4A1) is an orphan nuclear receptor that was identified as an immediate-early gene activated by multiple stressors [273]. Moreover, NR4A1 and other NR4A members (NR4A2; Nurr1; NR4A3; Nor1) are overexpressed and play a role in inflammatory- related diseases, including cancer, T-cell

exhaustion, fibrosis, and brain injury [213, 278, 280, 329-336]. NR4A1 regulates one or more of cancer cell proliferation, survival, cell cycle progression, migration, and invasion in various solid tumors (such as lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, and gastric cancer), and NR4A1 is effectively inhibited by bis-indole derived (CDIM) NR4A1 ligands which act as antagonists to suppress cancer progression [159, 160, 255, 283, 307]. For example, NR4A1 knockdown and CDIM/NR4A1 antagonists treatment effectively suppressed the growth of endometrial cancer cell lines [307]. In addition, NR4A1 knockdown and CDIM/NR4A1 antagonist inhibited the growth of human endometriotic lesions isolated from endometriosis patients by inhibiting NR4A1-regulated proliferation, mTOR signaling, and fibrosis in vitro [255]. CDIM/NR4A1 antagonist also suppressed the growth of endometriotic lesions in mice with endometriosis. Suggesting that NR4A1 plays an essential role in endometriosis progression and is a new therapeutic target for the non-hormonal treatment of endometriosis. Flavonoids are phytochemicals that naturally exist in plants, fruits, vegetables, and leaves and these compounds have many medicinal benefits, such as anticancer, antioxidant, anti-inflammatory, antiviral, neuroprotective, and cardio-protective properties [337-344]. Interestingly, the flavonoid kaempferol inhibits hepatic gluconeogenesis and induces GLUT4 expression and glucose uptake in muscle cells like CDIMs and other NR4A1 ligands [158, 313, 345]. Recent studies also showed that the flavonoid kaempferol and CDIMs regulate expression of the histone methyltransferase G9a gene which was recently shown to be an NR4A1-dependent gene in Rh30 cells [283, 316]. A recent study showed that flavonoids quercetin and kaempferol bound

NR4A1 and acted as receptor antagonists to inhibit NR4A1-dependent pro-oncogenic pathways/genes in rhabdomyosarcoma cells [276]. Since NR4A1 plays a critical role in endometriosis, this study has investigated the potential applications of the flavonoids quercetin and kaempferol as nutraceuticals for treatment of endometriosis.

4.2. Materials and methods

4.2.1 Mice models

Luciferase-expressing FVB [Tg(CAG-luc, GFP)L2G85Chco] and FVB female mice were purchased from Jackson Laboratory and maintained in the designated animal care facility at Baylor College of Medicine according to the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use of laboratory animals. An IACUC-approved protocol was followed for all animal experiments in this study.

Primary human endometriotic cells from endometriosis patients

Using our primary human endometrial stromal cells isolated from an ovarian endometrioma [255], we generated immortalized human endometriotic stromal cells using lentivirus expressing human telomerase reverse transcriptase [255, 346]. In addition, the immortalized human endometriotic epithelial cells generated from ovarian endometrioma cells were employed [347]. Primary normal endometrial stromal cells (NESCs) were isolated from the biopsy of the eutopic endometrium of normal women (NEM) in the proliferative phase [255]. Ishikawa cells were purchased from Millipore Sigma (Catalog number: 99040201). These cells were maintained in DMEM/F12 growth

medium supplemented with 10% FBS and 1× antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO). All cells were incubated at 37℃ in CO2 incubator in an atmosphere of humidified 5% CO2 and 95% air. The short tandem repeat analysis (STR) validated that these cells were not contaminated with another type of cells. In addition, the identity of human endometrial epithelial and stromal cells was validated by the expression of cytokeratin 18 and vimentin by Western blot analyses, respectively.

4.2.2 Reagents and antibodies

The primary antibodies used were epidermal growth factor receptor (EGFR; 4267), survivin (2808), Sestrin 2 (8487), p-mTOR (2971), mTOR (2972), p-S6RP (2215), S6RP (2217), p-4E-BP1 (9451), 4E-BP1 (9644), α-smooth muscle actin (SMA; 19245) from Cell Signaling Technology (Danvers, MA, US); c-Myc (sc-40) from Santacruz Biotechnology (Santacruz, CA, US); NR4A1 (LS-C118011) antibody was purchased from LSBio (Seattle, WA); β-actin (A1978) from Sigma Aldrich Corporation (Milwaukee, WI, US); TXNDC5 (GTX106914), COL1A1 (GTX112731), CTGF (GTX124232), and fibronectin (FN; GTX112794) from GeneTex, Inc. (Irvine, CA, US). Secondary antibodies for rabbit (7074), mouse (7076), and anti-rabbit Alexa Fluor® (4412) were purchased from Cell Signaling Technology (Danvers, MA, US). Two siNR4A1 oligonucleotides used in this study were 5’-CAGUGGCUCUGACUACUAU-3’ (1) and 5’-GAGAGCUAUUCCAUGCCUA-3’ (2) and nontargeted scrambled small interfering ribonucleic acids (siRNA; iGL2) were used as a control from Sigma Aldrich

Corporation (The Woodlands, TX, US). Quercetin and kaempferol were purchased from Indofine (Hillsborough, NJ).

4.2.3 Cell proliferation assay

Patient-derived endometriotic cells IHESC, IHEEC, human endometrial adenocarcinoma cell Ishikawa and normal cell NEM were seeded into a 96-well plate. The cells were treated for 24 hours with either dimethyl sulfoxide (DMSO) or different concentrations of quercetin and kaempferol (25-100 μM or 25-150 μM). IHESC and IHEEC were treated with 50 nM of 2 siNR4A1 oligonucleotides to downregulate NR4A1; 50 nM of non-target siRNAs was employed as the control of siRNA. Afterward, the medium was removed, and the MTT solution diluted in phosphate-buffered saline (PBS) was added to cell cultures. After 3 hours of incubation, the medium was aspirated and washed with PBS. DMSO was added and incubated at 37°C for 10 minutes, and absorbance was measured at 570 nM.

4.2.4 Western blotting

IHESC, IHEEC and Ishikawa cells (2×10^5) were seeded and allowed to attach for 24 hours, and cells were then treated for 24 hours with either DMSO or different concentrations of quercetin and kaempferol. Cells were then lysed, and whole-cell lysates were resolved in 10% SDS-PAGE gels. Proteins were transferred using polyvinylidene fluoride membrane by wet blotting followed by primary and secondary

antibody incubation and detected using enhanced chemiluminescence reagent as previously described. Immunofluorescence IHEEC and Ishikawa cells were seeded in Nunc chambered coverglass followed by various drug treatments. The cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at 37°C. Cells were then blocked and incubated overnight with primary α-SMA antibody in the buffer (5% bovine serum albumin in PBS) at 4°C, followed by Alexa Fluor®–conjugated secondary antibody at a dilution of 1:250 for 2 hours at room temperature. Finally, cells were observed using a Zeiss confocal fluorescence microscope.

4.2.5 RNA interference

IHESC, Ishikawa, and IHEEC cells were seeded in 6-well plates and allowed to grow to 60% confluence (24 hours), and then transfections were performed with Lipofectamine 2000 according to the manufacturer's protocol. Both siNR4A1 oligonucleotides and nontargeted control siRNAs were used. Six hours after transfection, the medium was replaced with fresh medium and left for 72 hours, and then the cells were harvested, and protein expression was determined. NR4A1 knockdown efficiency by NR4A1 siRNA was determined by Western blotting.

4.2.6 Determination of the intrinsic transcriptional activity of NR4A1 upon flavonoids exposure

IHESC, Ishikawa, and IHEEC cells were plated on 12-well plates in DMEM/F12 supplemented with 2.5% charcoal-stripped fetal bovine serum. Cells were allowed to attach and grow for 12 hours, and various amounts of deoxyribonucleic acid (i.e., upstream activation sequence (UAS)x5-Luc [500 ng], GAL4-NR4A1 [50 ng] were co-transfected into each well using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, US) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with a plating medium containing either solvent (DMSO) or indicated concentrations of the quercetin and kaempferol for 18 hours. Cells were then lysed using freeze–thaw protocol, and cell extract was used for luciferase and b-gal assays. LumiCount (Packard) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against corresponding β-gal activity values and protein concentrations determined by Bradford assay. GAL4-NR4A1 constructs contain full-length NR4A1 coding sequences, and all the plasmids used in this study were previously described [160, 255, 276].

4.2.7. Computation-based molecular modeling

Molecular modeling studies were conducted using Maestro (Schrödinger Release 2020-1, Schrödinger, LLC, New York, NY, 2020). The version of Maestro used for these studies is licensed to the Laboratory for Molecular Simulation (LMS), a Texas A&M University core user facility for molecular modeling and is associated with the Texas

A&M University High Performance Research Computing (HPRC) facility (College Station, TX, 77843). All Maestro- associated applications were accessed via the graphical user interface (GUI) VNC interactive application through the HPRC Ada OnDemand portal. The crystal structure coordinates for human orphan nuclear receptor NR4A1 ligand binding domain (LBD) [158] were downloaded from the Protein Data Bank (https://www.rcsb.org; PDB ID 3V3Q). The human NR4A1 LBD crystal structure was prepared for ligand docking utilizing the Maestro Protein Preparation Wizard; restrained minimization of the protein structure was performed utilizing the OPLS3e force field. Each ligand (quercetin and kaempferol) three-dimensional structure was prepared for docking utilizing the Maestro LigPrep, again using the OPLS3e force field. Maestro Glide [276, 277, 348] was utilized with the default settings to dock each prepared ligand to the prepared protein, predict the lowest energy ligand binding orientation, calculate the predicted binding energy in units of kcal/mol, and determine specific amino acid/ligand interactions.

4.2.8. Noninvasive reporter mouse model of endometriosis (NREN)

To test drug effects on endometriosis, a new NREN was generated by using luciferasereporter mice [FVB-Tg(CAG-Luc, GFP)L2G85Chco/J, Jackson Laboratory, catalog #: 008450]. Under anesthesia, uterine horns were isolated from a female luciferase reporter mouse (8 weeks old) at the estrus stage. The isolated horns were opened longitudinally in a petri dish. The endometrial tissues were scraped away from the myometrial layer using a scalpel to ensure that only endometrial cells without

myometrial and perimetrical tissues were injected to mimic the menstrual dissemination of endometrial tissues. The scraped endometrial mass was suspended in PBS buffer, and 1 $x10^6$ cells were injected intraperitoneally into one recipient ovariectomized syngeneic female mouse (8 weeks old, FVB) implanted with an estrogen pellet (3.6 mg, 60 days lease) to induce endometriosis [255].

4.2.9. Endometriosis treatment with kaempferol and quercetin

Endometriosis was induced in mice with the NREN method. After ectopic lesions were established in mice (3rd week after endometriosis induction), we randomly divided mice with endometriosis into three groups and then intraperitoneally injected mice with vehicle (5% DMSO and 10% 2-hydroxypropyl-β-cyclodextrin), 100 mg/kg of kaempferol and 100 mg/kg of quercetin (once a day, for 14 days). After drug treatment, we determined the luciferase activity of the ectopic lesions treated with vehicle, kaempferol, and quercetin using in vivo image system.

4.2.10. Quantifying bioluminescence data

Mice were anesthetized with a 1.5% isoflurane/air mixture using an Inhalation Anesthesia System (VetEquip). Next, d-Luciferin (ThermoFisher, catalog number: L2916) was intraperitoneally injected at 40 mg/kg mouse body weight. Ten minutes after the D-luciferin injection, the mice were imaged using an IVIS Imaging System (Xenogen) with continuous 1% to 2% isoflurane exposure. Imaging variables were

maintained for comparative analysis. Grayscale-reflected and pseudocolorized images reflecting bioluminescence were superimposed and analyzed using Living Image software (Version 4.4, Xenogen). A region of interest (ROI) was manually selected over the relevant signal intensity regions. The area of the ROI was kept constant across experiments, and the intensity was recorded as total photon counts per second per cm2 within the ROI.

4.2.11. Statistical analysis

All experiments have been repeated a minimum of three times. The data are expressed as the mean ± standard error (SE). A one-way analysis of variance was used to determine statistical significance. P values<0.05 were considered statistically significant.

4.3. Results

4.3.1. Kaempferol and quercetin are NR4A1 antagonists.

The three-dimensional interactions of kaempferol and quercetin with the ligand binding domain (LBD) of NR4A1 and analysis which measures quenching of intrinsic tryptophan fluorescence revealed that quercetin (Kd = 0.93 μmol/L) had a higher binding affinity to the LBD of NR4A1 than kaempferol (Kd = 3.1 μmol/L) [276]. The Schrodinger/Maestro modelling showed that quercetin bound to positive amino acids (Glu109, Phe112, Leu113, Glu114, and Ile260) marked as a yellow dashed line and negative amino acids (Thr236, Leu239, Ile260, and Thr264) drawn with orange dashed

lines in LBD of NRA41 (Fig. 4-1A). Kaempferol also bound to positive amino acids (Glu109, Ser110, Ala111, Phe112, Leu113, Glu114, and Ile260) and negative amino acids (Thr236, Leu239, Ile260, and Thr264) in LBD of NR4A1 (Fig. 4-1B). Therefore, both quercetin and kaempferol have a similar binding orientation within the LBD binding pocket of NR4A1.

Next, we determined how the binding of quercetin and kaempferol changes NR4A1 activity. To determine the intrinsic transcriptional activity of NR4A1, the expression vector for GAL4 DNA binding domain fused to NR4A1 (Gal4 NR4A1) and the luciferase reporter plasmid containing five copies of Gal4 binding upstream activation sequence (UAS) were co-transfected into immortalized human endometriotic epithelial cells (IHEECs), immortalized human endometriotic stromal cells (IHESCs), and Ishikawa cells. Compared to vehicle treatment, both quercetin and kaempferol treatment decreased the intrinsic transcriptional activity of NR4A1 in a dose-dependent manner in all tested endometrial cells (Figs. 4-1C, 4-1D, and 4-1E). Therefore, quercetin and kaempferol are natural flavonoids that directly bind to NR4A1 to decrease its transcriptional activity.

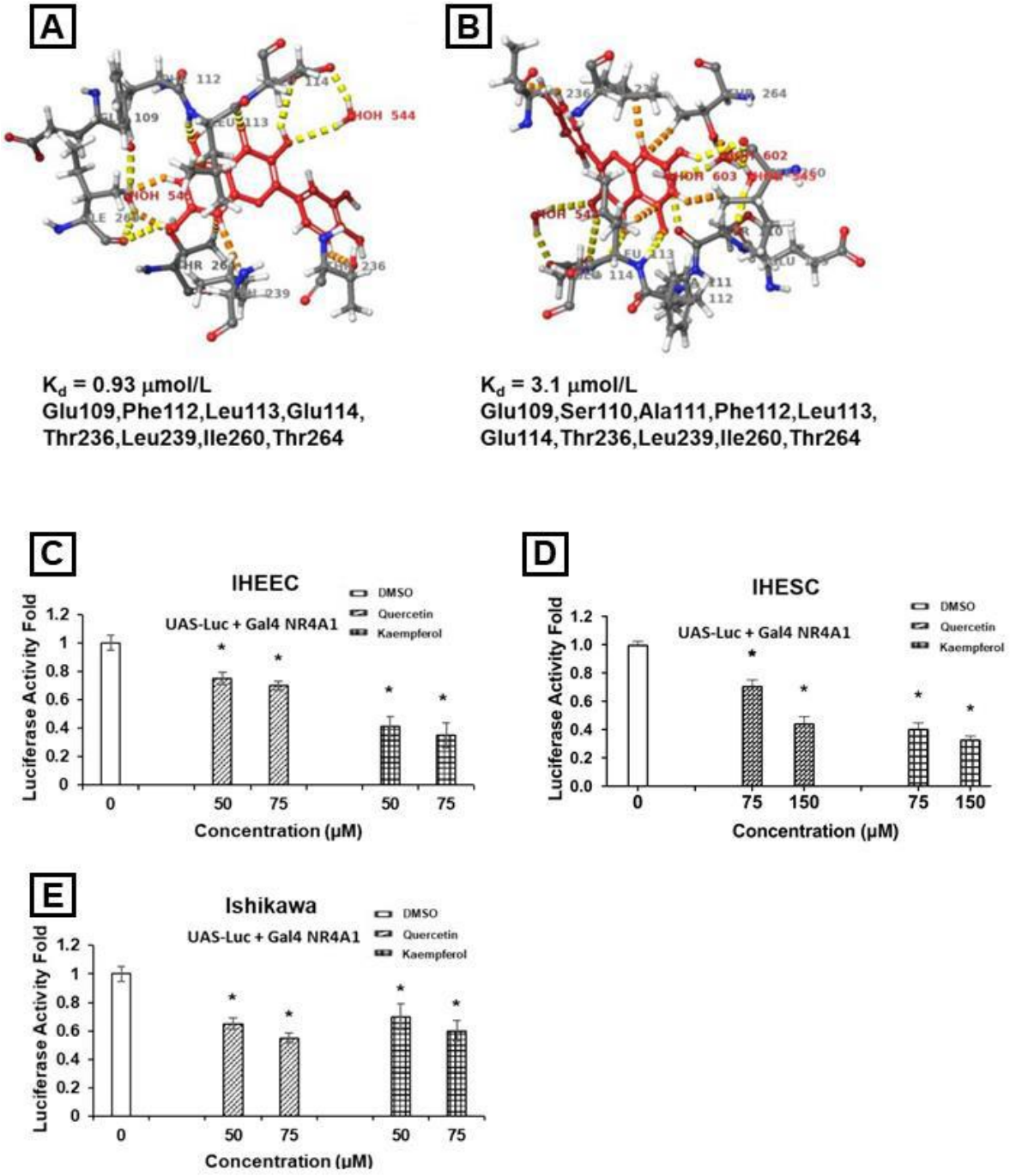


Figure 4-1. Kaempferol and quercetin as NR4A1 ligands. Quercetin (A) and kaempferol (B) interactions with the LBD of NR4A1 using a modeling approach (42-44) and critical interacting amino acids are indicated. Effects of kaempferol and quercetin on luciferase activity were determined in IHEEC (C), IHESC (D), and Ishikawa (E) cells transfected with a chimeric GAL4 NR4A1 construct and a UAS-luc reporter gene. Luciferase activity was determined as outlined in the Methods. Results (C-E) are expressed as means ± SE for at least 3 determinations, and significant ($p<0.05$) changes compared to DMSO (control) are indicated (*).

4.3.2. Quercetin and kaempferol suppressed the growth of human endometriotic cells but not normal endometrial cells.

To define whether NR4A1 has an essential role in the progression of IHEEC, IHESC and Ishikawa cells, NR4A1 levels in these cells were decreased by transfection with 2 different siRNAs against NR4A1. Ishikawa endometrial cancer cells are routinely used in endometriosis studies and were included in this research. As the siRNA control, non-targeting siRNA (siCtrl) was employed. Compared to siCtrl, NR4A/siRNA effectively reduced NR4A1 protein levels in IHEEC, IHESC, and Ishikawa cells (Fig. 4-2A) and then suppressed proliferation of IHEEC, IHESC, and Ishikawa cells (Fig. 4-2B – 4-2D). Therefore, NR4A1 is essential in human endometriotic epithelial and stromal cell proliferation. Next, we determined whether inhibition of NR4A1 activity by quercetin and kaempferol also suppressed growth of human endometriotic cells. Both quercetin and kaempferol suppressed proliferation of IHEECs and IHESCs in a dose- dependent manner (Figs. 4-2E and 4-2F). Furthermore, in addition to human endometriotic cells, quercetin and kaempferol treatment also suppressed the proliferation of Ishikawa cells (Fig. 4-2G). In contrast, the proliferation of normal endometrium from women without endometriosis (NEM) was not inhibited by quercetin and kaempferol (Fig. 4-2H). Therefore, quercetin and kaempferol specifically inhibited growth of human endometriotic cells from endometriosis patients but not normal endometrial cells.

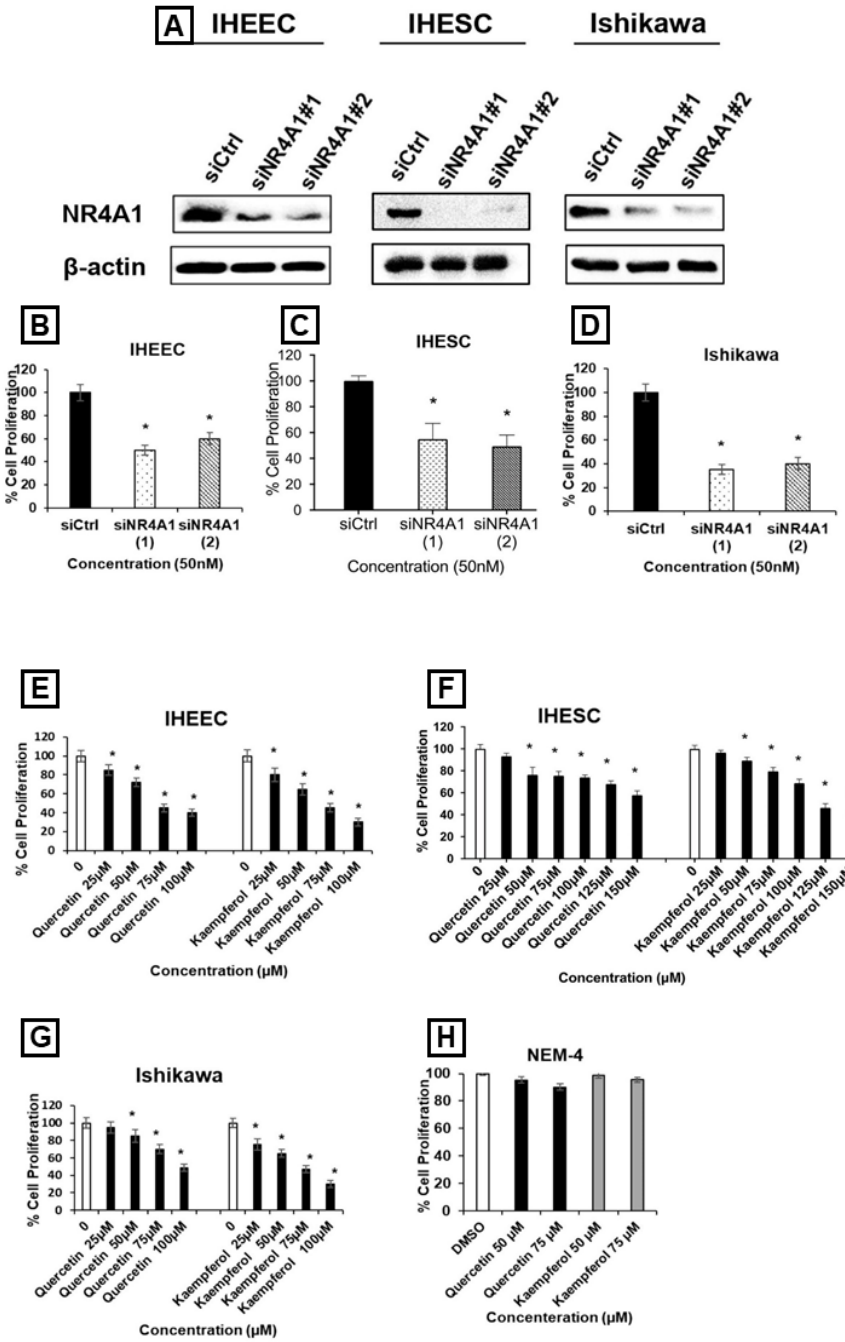


Figure 4-1. Endometriotic cell growth inhibition by quercetin and kaempferol. (A) Knockdown efficiencies of NR4A1 in IHEEC, IHESC, and Ishikawas cell determined by Western blots of whole cell lysates as outlined in the Methods. (B-D) IHEECs (B), IHESCs (C), and Ishikawa cells (D) were transfected with two oligonucleotides targeting NR4A1, and levels of NR4A1 were determined with Western blot analysis. (E-G) The proliferation of IHEEC (E), IHESC (F), and Ishikawa (G) cells transfected with siNR4A1 oligonucleotides and control siRNA (siCtrl) was determined by the MTT assays. (E-H) The proliferation of IHEEC (E), IHESC (F), Ishikawa (G), and NEM-4 (H) cells treated with quercetin or kaempferol for 24 hours was determined by MTT assays. Results are expressed as means ± SE for at least 3 replicate determinations significant ($p<0.05$) inhibition of cell growth is indicated.

4.3.3. Quercetin and kaempferol decrease the EGFR/c-Myc/Survivin growth axis in human endometriotic cells.

NR4A1 regulates the expression of growth-promoting and survival genes (EGFR, c-Myc, survivin) in endometriotic and endometrial cancer cells [255, 307]. Thus, we determined whether quercetin and kaempferol also block the growth survival signaling in human endometriotic cells. The knockdown of NR4A1 decreased EGFR, c-Myc, and survivin levels in IHEECs compared to control siRNA (Fig. 4-3A). Also, quercetin and kaempferol treatment decreased levels of EGFR, c-Myc, and survivin in IHEEC cells compared to the vehicle. Therefore, quercetin and kaempferol treatment effectively suppressed the NR4A1-mediated EGFR/c-Myc/Survivin axis in IHEECS to inhibit the growth of these cells. Compared to IHEECs, the NR4A1 knockdown and quercetin, and kaempferol reduced levels of EGFR and survivin compared to their control (Fig. 4-3B). However, c-Myc levels were not reduced by NR4A1 knockdown in IHESCs and quercetin, and kaempferol treatment increased c-Myc in IHESCs compared to the vehicle (Fig. 4-3B). In human endometriotic stromal cells, therefore, NR4A1 did not regulate the c-MYC expression. The knockdown NR4A1 and quercetin and kaempferol treatment effectively suppressed levels of EGFR, c-Myc, and survivin in Ishikawa cells as also observed in IHEECs (Fig. 4-3C). Therefore, quercetin and kaempferol treatment suppress the NR4A1-mediated EFGR/c-Myc/survivin axis in human endometriotic epithelial but not in human endometriotic stromal cells.

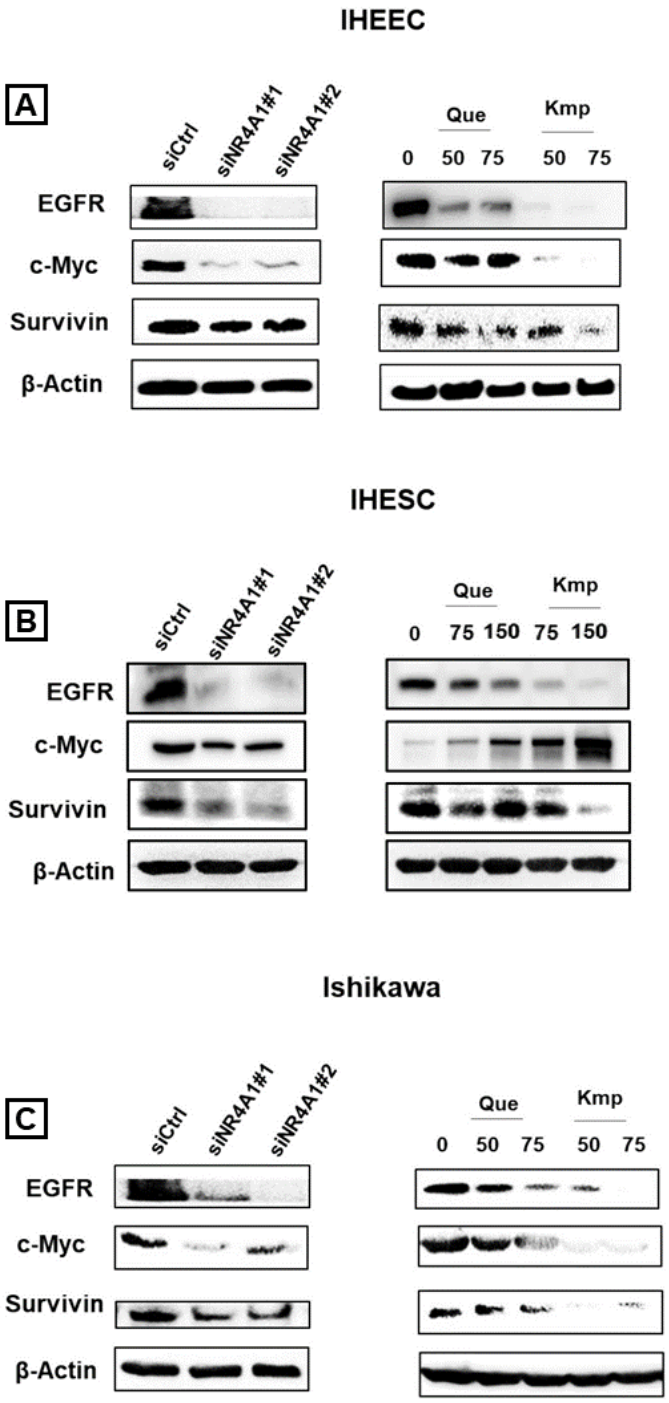


Figure 4-1. siNR4A1 knockdown, quercetin, and kaempferol decrease expression of growth-promoting and survival genes. (A-C) The levels of EGFR, c-Myc, and Survivinin IHEEC (A), IHESC (B), and Ishikawa (C) cells transfected with siNR4A1 oligonucleotides or treated with quercetin or kaempferol for 24 hours were analyzed by Western blotting.

4.3.4. Quercetin and kaempferol treatment increased the ER oxidative/ER stress by reduction of TXNDC5 in human endometriotic cells.

NR4A1 knockdown or treatment with NR4A1 antagonists induced oxidative/ER stress by decreasing TXNDC5 and elevating levels of the oxygen sensor protein setrin2 (SESN2) in Ishikawa cells [307]. To validate whether quercetin and kaempferol treatment also induced similar responses in human endometriotic cells, expression levels of TXNDC5 and SESN2 were examined in IHEECs and IHESCs after treatment with these flavonoids. NR4A1 knockdown and quercetin and kaempferol treatment decreased levels of TXNDC5 and increased SESN2 in IHEEC compared to their control (Fig. 4-4A). NR4A1 knockdown also decreased TXNDC5 expression and increased SESN2 to enhance oxidative/ER stress (Fig. 4-4B). In contrast, kaempferol and quercetin treatment induced SESN2 without reducing TXNDC5 expression in IHESCs (Fig. 4-4B). Therefore, kaempferol and quercetin treatment induced the oxygen-sensing SENS2 in IHESC cells independent of TXNDC5 suggesting a role for other NR4A1-regulated pro-reductant genes and these are currently being investigated. NR4A1 knockdown, kaempferol and quercetin induced also decreased TXNDC5 and induced SESN2 levels in Ishikawa cells as observed for IHEECs (Fig. 4-4C). To determine whether induction of ROS associated with downregulation of TXNDC5 induced SESN2, changes in levels of SESN2 were determined in human endometriotic cells and Ishikawa cells in the presence or absence of 5 mM glutathione (GSH). GSH treatment alone did not affect SESN2 levels but significantly inhibited induction of SESN2 in IHEECs, IHESCs, and Ishikawa cells treated with quercetin and kaempferol alone (Fig. 4-4D).

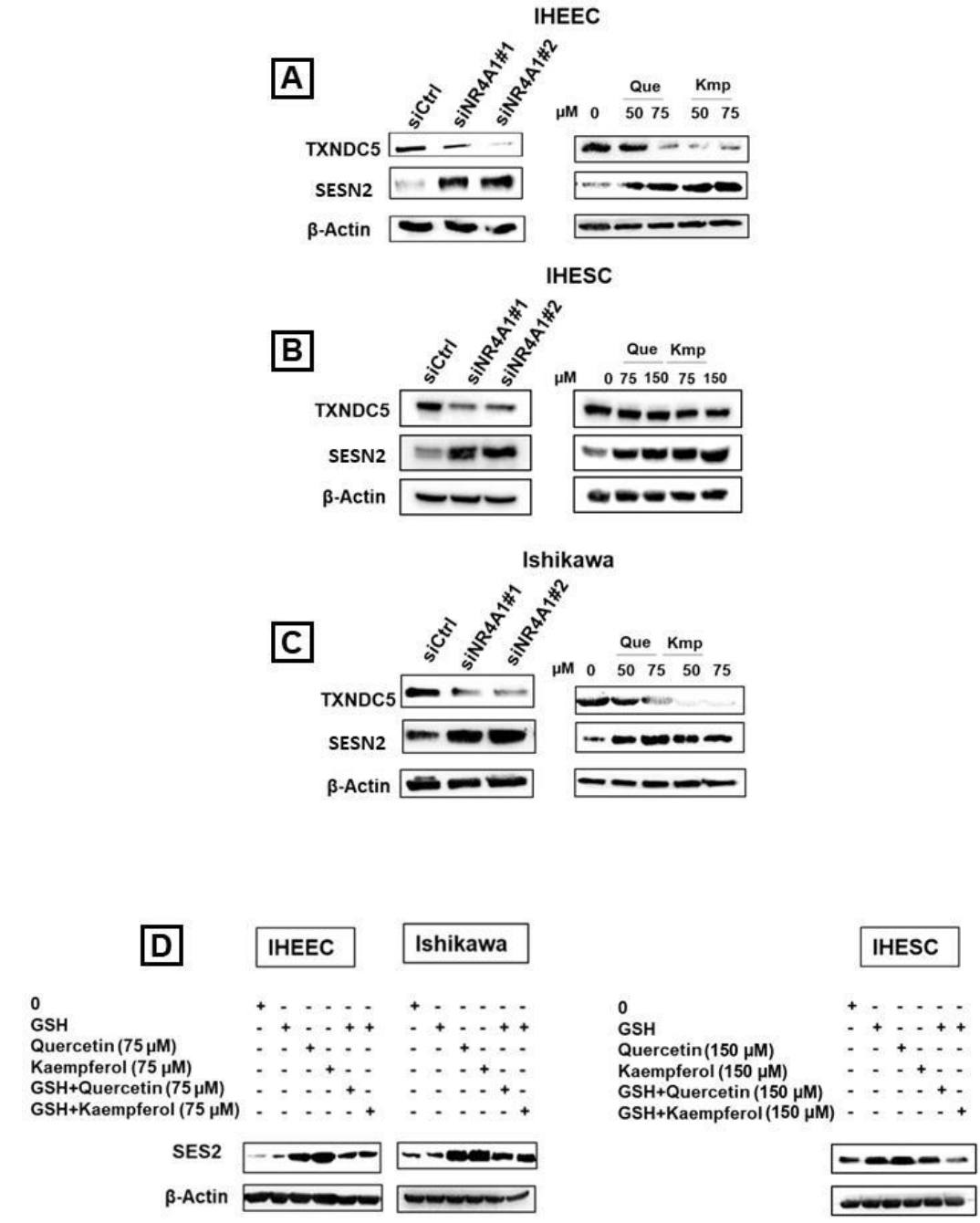


Figure 4-1. siNR4A1, quercetin, and kaempferol modulate oxidative/ER stress pathway genes in endometriotic cells. (A-C) Levels of TXNDC5, SESN2, β-Actin in IHEEC (A), IHESC (B), and Ishikawa (C) cells transfected with siNR4A1 oligonucleotides or treated with quercetin or kaempferol for 24 hours were determined by Western blot analysis. (D) Levels of SESN2 and β-Actin in IHEEC, IHESC, and Ishikawa cells were treated with DMSO, GSH, quercetin, kaempferol, GSH plus quercetin, and GSH plus kaempferol for 24 hours were determined by Western blotting.

4.3.5. Quercetin and kaempferol inhibited mTOR signaling in human endometriotic cells.

The induction of SESN2 inhibits mTOR signaling in various cancer cells [160, 307] and therefore, we determined whether quercetin and kaempferol also inhibited the SESN2-mediated mTOR signaling in human endometriotic cells. NR4A1 knockdown, quercetin, and kaempferol treatment significantly decreased phosphorylated (p)-mTOR, p-S6RP, and p-4EBP1 in both IHEECs and IHESCs compared to their control (Fig. 4-5A and 4-5B). NR4A1 knockdown and quercetin and kaempferol treatment also significantly inhibited mTOR signaling pathways by reducing p-mTOR, p-S6RP, and p-4EBP1 in Ishikawa cancer cells (Fig. 4-5C). Thus, quercetin and kaempferol effectively suppressed the mTOR signaling by increasing SESN2 in human endometriotic cells and this is consistent with their growth inhibitory effects (Fig. 4-2).

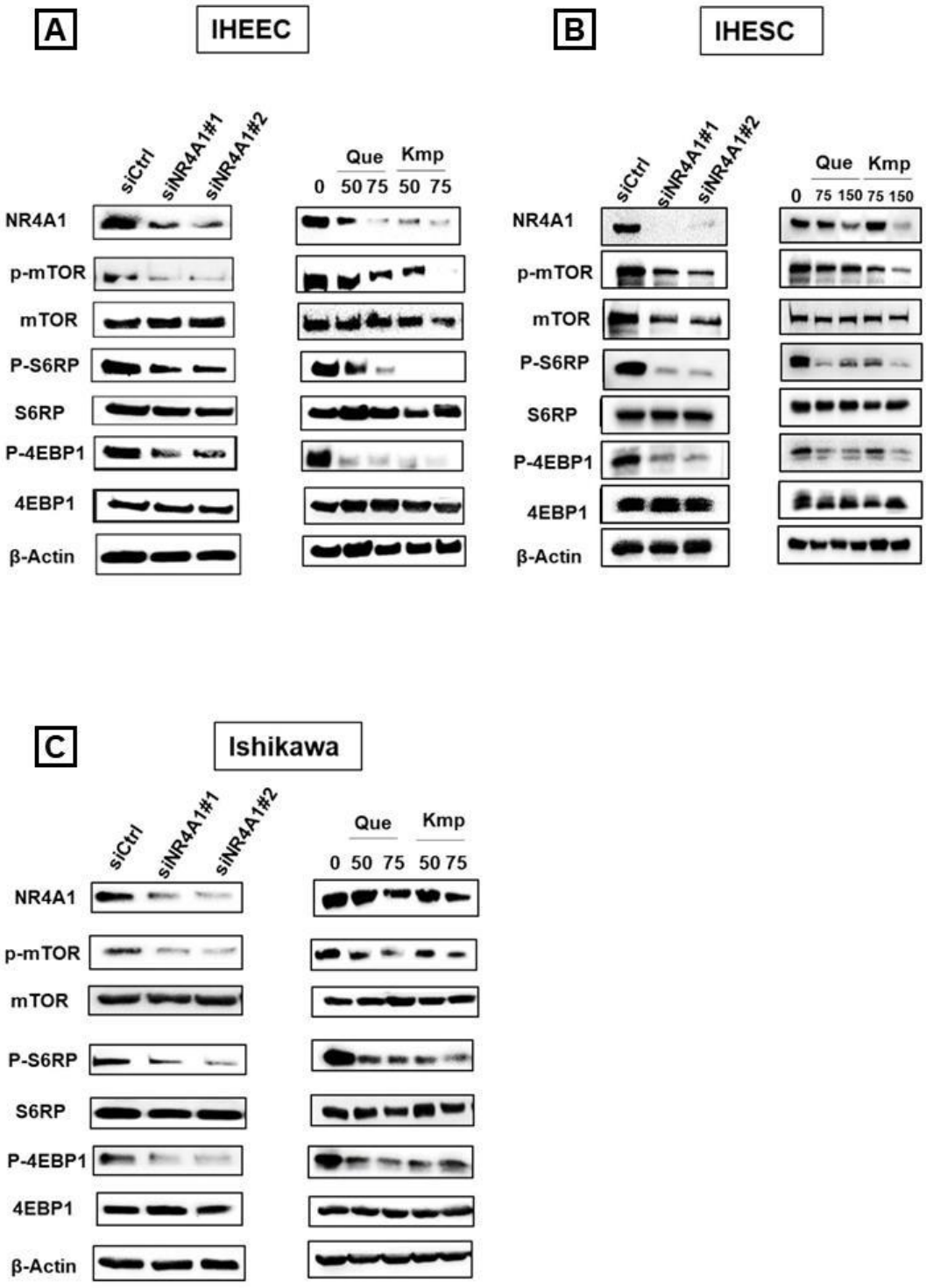


Figure 4-1. siNR4A1, quercetin, and kaempferol inhibit mTOR signaling in human endometriotic cells. (A-C) Levels of mTOR marker proteins in IHEEC (A), IHESC (B), and Ishikawa (C) cells transfected with siNR4A1 oligonucleotides or treated with kaempferol or quercetin for 24 hours were determined by Western blotting.

4.3.6. Quercetin and kaempferol inhibited fibrosis progression in human endometriotic cells.

The fibrosis progression mediated by α-smooth muscle actin (α-SMA) and other profibrotic genes has a critical role in endometriosis, and NR4A1 regulates the expression of profibrotic genes in human endometriotic cells [255]. The bis-indole-derived NR4A1 antagonist significantly reduced levels of α-SMA, fibronectin (FN), corrective tissue growth factor (CTGF) and collagen type 1 α-1 (COL1A1) in human endometriotic cells [255]. Therefore, we determined whether quercetin and kaempferol also suppressed fibrosis progression in human endometriotic cells. NR4A1 knockdown and quercetin and kaempferol treatment decreased expression of the fibrotic genes α-SMA, CTGF, COL1A1, and FN in IHEEC cells compared to their control (Fig. 4-6A). In contrast, NR4A1 silencing decreased level of FN, but did not reduce levels of α-SMA, COL1A1, and CTGF in IHESCs compared to control siRNA (Fig. 4-6B). Treatment with quercetin and kaempferol also did not decrease α-SMA, but decreased CTGF and FN in IHESCs compared to the vehicle (Fig. 4-6B). Whereas kaempferol but not quercetin decreased COL1A1 levels in IHESCs (Fig. 4-6B). Therefore, NR4A1 does not have an essential role in fibrosis progression in IHESCs. The NR4A1 knockdown, quercetin, and kaempferol treatment reduced levels of profibrotic genes in Ishikawa cells compared to their control (Fig. 4-6C). Immunofluorescence analysis also validated the reduction of α-SMA in IHEEC and Ishikawa cells by quercetin and kaempferol compared to the vehicle (Figs. 4-6D and 4-6E).

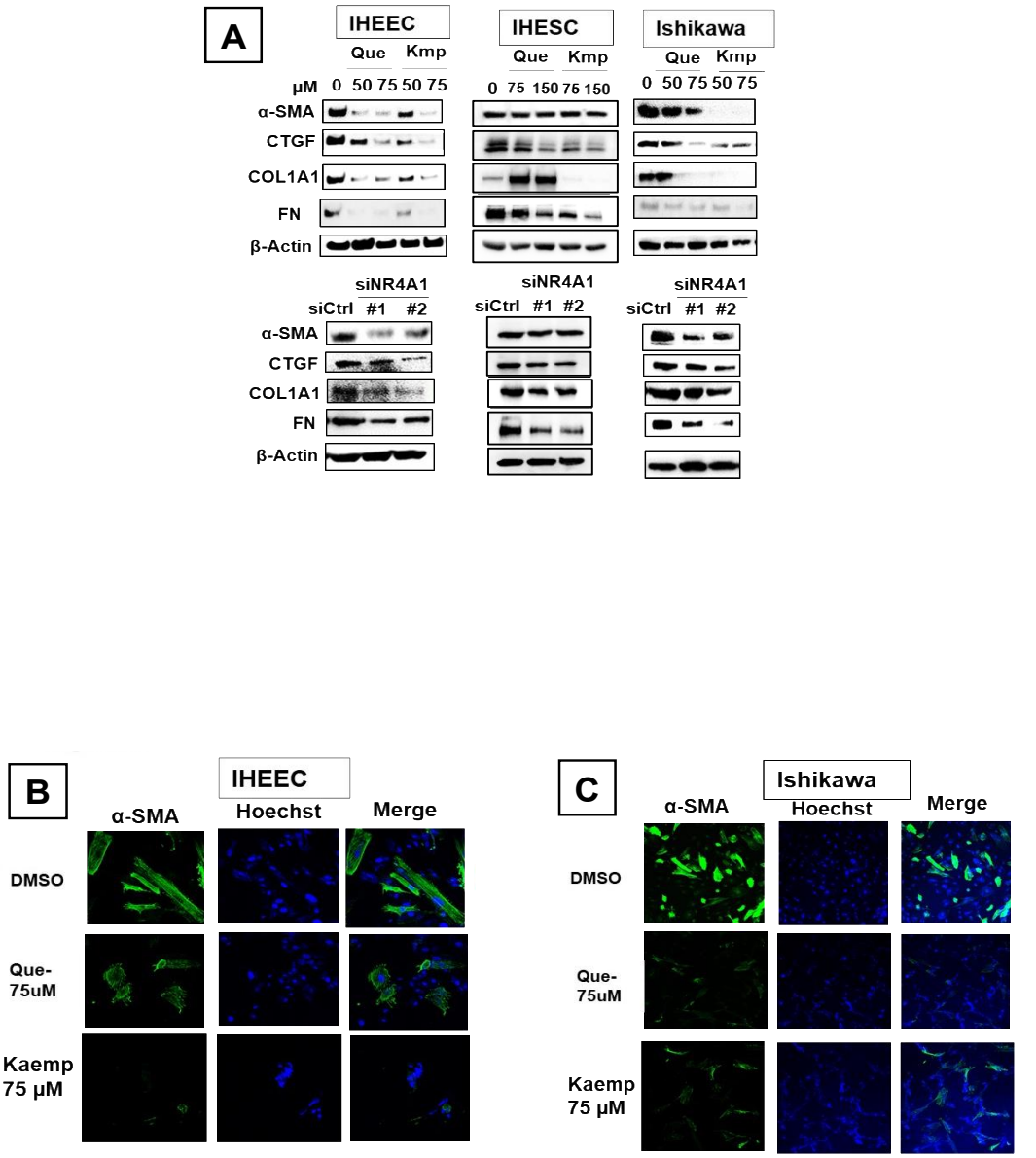


Figure 4-1. siNR4A1, quercetin and kaempferol inhibit fibrosis in human endometriotic cells. (A-C) Levels of fibrosis marker proteins in IHEEC (A), IHESC (B) and Ishikawa cells (C) treated with quercetin or kaempferol or transfected with siRNAs were determined by Western blotting. (D-E). Levels of α-SMA in IHEEC (D) and Ishikawa (E) cells treated with 75 μM quercetin and kaempferol for 24 hours were determined by immunofluorescence. Hoechst-stained nucleus.

4.3.7. Quercetin and kaempferol reduced the endometriosis progression in mice without toxicity.

To validate effects of quercetin and kaempferol on human endometriotic cells, we examined whether quercetin and kaempferol treatment suppress endometriosis progression in mice using the NERN method [255]. The luciferase activity analysis revealed that endometriotic lesions were well established in mice with endometriosis (Fig. 4-7A, 0 days after drug treatment). In the vehicle-treated mice, luciferase activities were elevated compared with vehicle treatment (Fig. 4-7A and 4-7B). Therefore, endometriotic lesions were continuously grown in mice with endometriosis and treated with a vehicle. Compared to the vehicle, however, quercetin (100 mg/kg/d) and kaempferol (100 mg/kg/d) significantly decreased luciferase activities of ectopic lesions compared to the before drug treatment (Fig. 4-7A and 4-7B). Therefore, quercetin and kaempferol treatment effectively suppressed the growth of endometriotic lesions in mice with endometriosis. Even though quercetin and kaempferol treatment reduced the growth of endometriotic lesions, quercetin, and kaempferol treatment did not affect body weights compared to vehicle (Fig. 4-7C). Thus, flavonoids quercetin and kaempferol safely antagonized multiple NR4A1-dependent pro-endometriotic responses in both in vitro and in vivo assays.

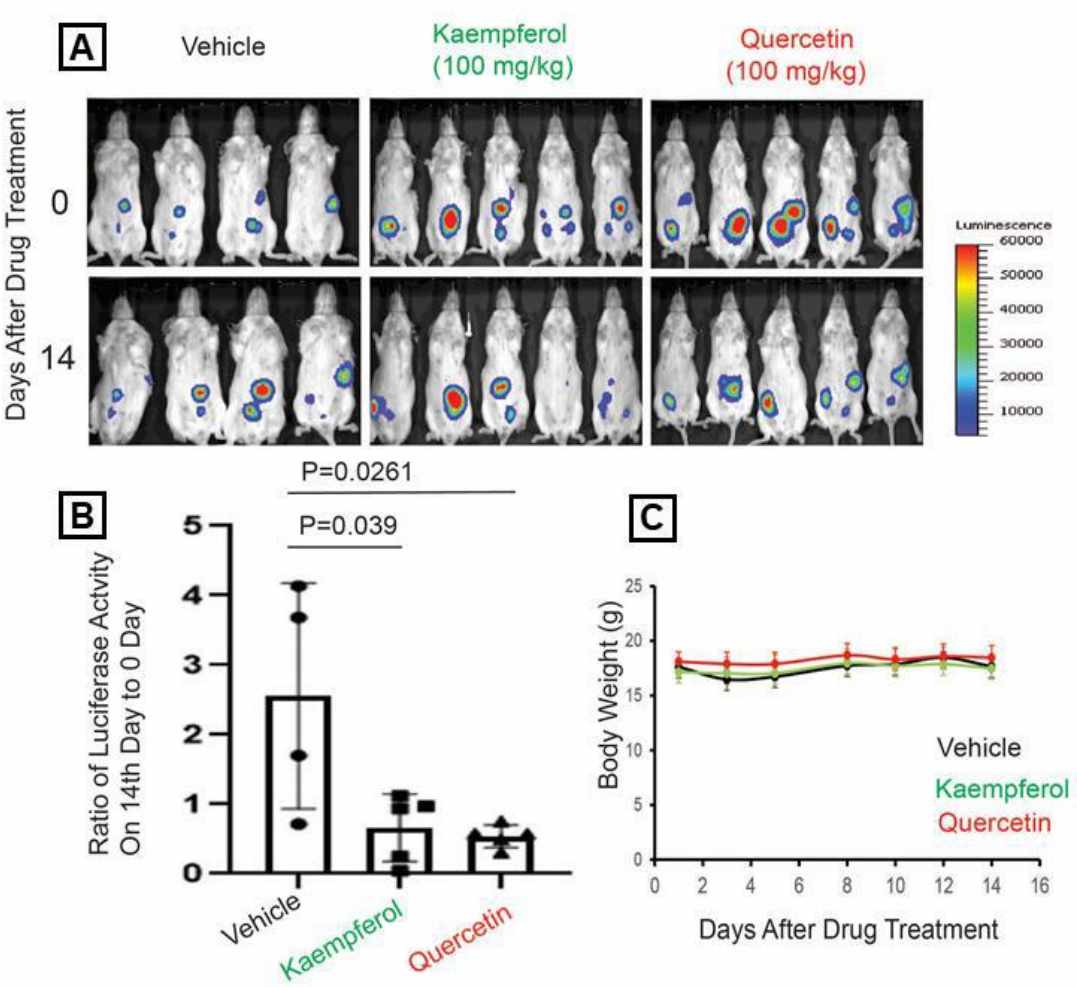


Figure 4-1. Quercetin and kaempferol treatment suppressed the growth of endometriotic lesions in mice with endometriosis. (A) Luciferase activity of ectopic lesions in mice with endometriosis treated with vehicle, quercetin, and kaempferol before drug treatment and 14th day after drug treatment. (B) Quantification of luciferase activity shown in Panel A. (C) The body weight changing of mice treated with vehicle, quercetin, and kaempferol for 14 days.

4.4. Discussion

NR4A1 and phosphorylated NR4A1 levels are elevated in endometriosis patients, and NR4A1 is involved in cell division, inflammation, programmed cell death, and fibrosis in endometriosis progression [170, 255]. Therefore, NR4A1 is considered a novel target for endometriosis treatment as a non-hormonal therapy to decrease the adverse effects of current hormonal therapies for endometriosis. In this context, bis-indole-derived compounds (CDIMs) have been identified as CDIM/NR4A1 antagonists effectively suppressed the growth of human endometrial cells derived from endometriosis patients by inducing apoptosis and inhibiting fibrosis pathways and also suppressed the growth of endometriotic lesions in mice with endometriosis [255, 307]. In addition to synthetic ligands, natural products have been applied for human disease treatment due to their beneficial therapeutic effects and relatively low toxicity [158, 338]. Interestingly, flavonoids also work as NR4A1 ligands to modulate NR4A1-mediated cellular pathways. For example, flavonoids kaempferol and quercetin are NR4A1 ligands that inhibit rhabdomyosarcoma cell growth by suppressing NR4A1-regulated oncogenic genes and cellular pathways [276]. Like CDIM/NR4A1 ligands, structurally diverse flavonoids such as quercetin, luteolin, myricetin, 3,6-dihydroxyflavone, chrysin, scutellarin, epigallocatechin-3-gallate, kaempferol, and flavonoid conjugates, also suppressed endometriosis progression by inhibiting cell growth, migration, and invasion in vivo and in vitro [342-344, 349-356]. These observations and our previous study [255] suggested that some flavonoids may also suppress endometriosis progression by inhibiting NR4A1-mediated cellular pathways.

How do flavonoids inhibit NR4A1? Like CDIMs, quercetin and kaempferol directly bind to NR4A1 with KD values of 0.93 and 3.1 μM, respectively, and inhibited the transcriptional activity of NR4A1 [276]. The binding site of quercetin and kaempferol in NR4A1 are similar to each other but not identical. For example, kaempferol, but not quercetin bound to Ser110 and Ala111 of NR4A1 with kaempferol and not quercetin. In contrast, interactions of quercetin and kaempferol were different from that previously reported by 1,1-bis(3'-indolyl)-1-(3,5-disubstitutedphenyl) methane analogs [274] suggesting that these NR4A1 ligands are selective receptor modulators, and this is consistent with their structure- and cell context- dependent differential effects on profibrotic genes (Fig. 4-6).

Quercetin and kaempferol are among the most ubiquitous polyphenols in fruit and vegetables. Kaempferol is safe for use, and quercetin supplements are added to the Food and Drug Administration's Generally Recognized as Safe list. Our mouse study also showed that quercetin and kaempferol treatment did not cause body weight loss, even though they suppressed the growth of endometriotic lesions (Fig. 4-7). In addition, quercetin and kaempferol have many other beneficial effects including lowering blood pressure and inflammation and potentially cardiovascular effects [337-344]. Therefore, dietary fruit and vegetables containing quercetin and kaempferol (such as broccoli, kale, dill, and spinach) should benefit endometriosis patients without any adverse effects compared to current hormonal therapy.

Generally, flavonoids are extensively metabolized in the liver and circulate in the blood as sulfate, methyl, and glucuronide conjugates [357, 358]. The three major plasma metabolites of quercetin were quercetin-3-sulfate, quercetin-30-sulfate, and quercetin-3-glucuronide. The major metabolite identified in plasma and urine was kaempferol-3-glucuronide [357]. However, the role of metabolites of quercetin and kaempferol in NR4A1 function in endometriosis is not elucidated. Therefore, the bioactivity and metabolism of quercetin and kaempferol metabolites in body tissues must be investigated to further understand the mechanism of action on NR4A1 targeted suppression of endometriosis.

Flavonoid-mediated NR4A1 inhibition studies have focused on the role of NR4A1 in survival pathways (such as PI3K/AKT, mTOR, oxidative/ER stress, and fibrosis) for the growth of endometriotic lesions. In addition, NR4A1 also has an essential role in immune cell function [359]. For example, NR4A1 has a critical role in regulatory T cell differentiation, and regulatory T cells have a crucial role in endometriosis progression [360, 361]. Thus, functional studies regarding the effects of flavonoid-targeted NR4A1 in immune cells will be needed to more fully understand the detailed molecular mechanism of how NR4A1 enhances endometriosis progression. Nevertheless, results of this study suggest that the nutraceutical applications of quercetin and kaempferol may be useful adjuncts for treating some symptoms of endometriosis.

CHAPTER V

BIS-INDOLE DERIVED COMPOUNDS AS DUAL RECEPTOR LIGANDS FOR NUCLEAR RECEPTOR 4A1 (NR4A1) AND NR4A2

5.1. Introduction

The nuclear receptor (NR) superfamily consists of 48 members which exhibit several common structural features including C- and N-terminal domains associated with activation function 2 (AF-2) and AF-1 respectively and a ligand binding region in the C-terminus [362-364]. Internal regions of NRs consist of a zinc finger DNA binding domain (DBD) and an adjoining hinge region. The NRs have been subdivided into different classes which include the steroid hormone and heterodimeric endocrine receptors, the lipid sensor and enigmatic adopted orphan receptors and the orphan receptors [362]. The orphan receptors include 14 members for which endogenous ligands have not yet been identified [365, 366]. Nevertheless, orphan nuclear receptors and most other NRs act as nuclear transcription factors that modulate gene expression through direct interaction with their cognate cis-elements or by interacting with other DNA bound transcription factors to coactivate their transcription. The different classes of NRs including orphan NRs play major roles in maintaining cellular homeostasis and pathophysiology and NRs are among the most important targets for clinically approved chemotherapeutics used to treat multiple diseases including cancer.

The nerve growth factor B (NGFB) or NR4A subfamily of orphan receptors include NR4A1 (Nur77, TR3), NR4A2 (Nurr1), and NR4A3 (Nor1) are early intermediate genes that respond to multiple stressors [273, 309, 367, 368]. NR4A bind as monomers and dimers to NBRE (AAGGTCA) and NuRE (TGATATTTACCTCCAAATGCCA) cis elements respectively and NR4A1 and NR4A2 also bind as heterodimers to the retinoid X receptor (RXR) [159, 369, 370]. Based on results of extensive biological and functional studies of wild type and NR4A-/- mice it was demonstrated that these receptors play important roles in the central nervous system, the response to multiple stimuli, inflammation, steroidogenesis, skeletal muscle function and metabolism [108, 172, 273, 308, 336, 367, 368, 371-378]. In addition, expression of NR4As are enhanced in multiple diseases and particularly those where there is elevated inflammation and stress.

Initial X-ray crystallographic studies showed that the LBD of NR4A2 did not exhibit a typical ligand binding space [179] consistent with its ligand-independent action however, subsequent studies show that several structurally diverse compounds bind NR4A1, NR4A2 and NR4A3 [157]. Although only a few compounds including prostaglandin A2 have been reported to bind NR4A3 [175, 379], several studies have identified small molecules that bind NR4A1 and NR4A2. NR4A1 ligands include cytosporone B and celastrol and related compounds, 1,1-bis(3’-indolyl)-(substituted phenyl) methane analogs, unsaturated fatty acids prostaglandin A2, 2-imino-6-methoxy-2H-chromene-3-carbothioimide, tetrandine, flavonoids, a bile acid metabolite, 12-

deacetyl-12-epi-scalaradial (a marine sponge product), nilotinib, resveratrol and other natural products [153, 157, 163, 164, 171-176, 312, 379-385]. Natural product and synthetic ligands for NR4A2 have also been identified and discussed in two recent reviews [386, 387]. NR4A2 ligands also include natural products, microbial and dopamine metabolites, prostaglandin A1 and E1, unsaturated fatty acids, drugs including statins, 4-aminoquinoline derivatives, bis-indole compounds and synthetic chemicals identified from screening chemical libraries [187, 198, 386-395]. There is evidence that the activities of NR4A1 ligands are structure and cell context-dependent [386, 393] and there are only a few reports indicating that individual compounds bind more than one receptor. Cytospone B is a well characterized NR4A1 ligand that also binds NR4A2 [386], and a recent report showed that prostaglandin A2 binds NR4A1, NR4A2 and NR4A3 [174, 175, 389]. Previous studies show that a series of 1,1-bis(3'-indolyl)-1-(3,5-disubstituted phenyl) methane (DIM-3,5) analogs were potent NR4A1 inverse agonists [274]. This study shows that these DIM-3,5- analogs also bind NR4A2 and represent a novel class of CDIMs that are dual NR4A1/2 ligands, which exhibit parallel NR4A1 and NR4A2 inverse agonist activities and inhibit NR4A1/NR4A2 pro-oncogenic pathways and genes.

5.2. Materials and methods

5.2.1. Ligand – receptor binding assays.

Isothermal titration calorimetry (ITC) was used to determine the ligand binding constant (Kd) to NR4A1 utilizing an Affinity ITC (TA Instruments, New Castle, DE). Briefly, the experimental setup was as follows. The ITC sample cell contained 250 μl of NR4A1 protein [ligand binding domain (LBD)] at a concentration of 20 μmol/l in buffer containing 20 mmol sodium phosphate/l (pH 7.4), 5% glycerol, and 1.0% ethanol. The ligand titrant was prepared in the same buffer as above at a ligand concentration of 66.6 μmol/l. The ligand titration into protein was performed at 25 °C with a stir rate of 125 rpm. Each ligand injection volume was 5 μl followed by 200 seconds to measure the total heat flow required to maintain constant temperature. A total of 20 injections were done for each ligand/NR4A1 combination. Each ligand titration into protein experiment was repeated for a total of three separate and independent experiments to generate the curves shown in the figure. In a separate set of injections, the same ligand was injected into buffer only (no protein) to determine heat flow as a result of ligand dilution into buffer. The ligand/buffer values were subtracted from the ligand/ protein values prior to data analysis using the Affinity ITC manufacturer-supplied data analysis software package. Sigmoidal curve fitting was performed using the Affinity ITC manufacturer-supplied data analysis software package to determine the following binding parameters: Kd, the equilibrium binding dissociation constant (μmol/l); n, the equilibrium ligand-to-protein binding stoichiometry (mol ligand per mol NR4A1); and ΔG, the equilibrium

free energy of ligand binding (kJ/mol). The resulting data are plotted as heat flow/area data (μJ) versus the cumulative resveratrol concentration μmol/l) present in the sample cell. Statistical analysis of the triplicate data was performed utilizing SigmaPlot 14.5 (Systat Software, Inc.) to determine the parameter mean (Kd, n, ΔG) and standard deviation. In addition, we also used a direct binding assay by determining the loss of fluorescence of a tryptophan residue in the LBD as previously described [210].

5.2.2. Computation-Based Molecular Modeling.

Molecular modeling studies were conducted using Maestro (Schrödinger Release 2020-1, Schrödinger, LLC, New York, NY, 2020). The version of Maestro used for these studies is licensed to the Laboratory for Molecular Simulation, a Texas A&M University core user facility for molecular modeling and is associated with the Texas A&M University High Performance Research Computing facility. All Maestro-associated applications were accessed via the graphical user interface (GUI) VNC interactive application through the HPRC Ada OnDemand portal. The crystal structure coordinates for human orphan nuclear receptor NR4A1 ligand binding domain (LBD) [158] were downloaded from the Protein Data Bank (https://www.rcsb.org; PDB ID 3V3Q). The human NR4A1 LBD crystal structure was prepared for ligand docking utilizing the Maestro Protein Preparation Wizard; restrained minimization of the protein structure was performed utilizing the OPLS3e force field. Each ligand (resveratrol or DIM-3,5-Cl2) three-dimensional structure was prepared for docking utilizing the Maestro LigPrep, again using the OPLS3e force field. Maestro Glide [276, 277] was used with

the default settings to dock each prepared ligand to the prepared protein, predict the lowest energy ligand binding orientation, and calculate the predicted binding energy in units of kcal/mol.

5.2.3. Cell culture, reagents, and antibodies.

RKO, SW480 and HCT116 colon cancer cells are purchased from American Type Culture Collection (Manassas, VA). Cells are cultured in DMEM medium with 10% FBS at 37 °C in the presence of 5% CO2. The details of antibodies used for Western blots and for chromatin immunoprecipitation (ChIP) assays are summarized in Supplemental Table 1.

5.2.4. Cell proliferation assay

Cell proliferation was investigated using XTT Cell Viability Kit (Cell Signaling Biotechnology) according to the manufacturer's instructions. Cells (1.5×10^4/well) were plated in 100 μl of plating medium (as above) in 96-well plates and allowed to attach for 24 hours. The medium was then changed to DMEM containing 2.5% charcoal-stripped FBS, and either vehicle (dimethyl sulfoxide (DMSO)) or designed concentrations of compounds in DMSO were added. After 24 and 48 hours of culture, 35 μL of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl) 3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the each well. The optical density was read at

450 nm wavelength in a plate reader after 4 hours of incubation. All determinations were replicated in at least three separate experiments.

5.2.5. Transfection and luciferase assay

Cells were plated on 12-well plates at 5×10^4/well in DMEM medium supplemented with 2.5% charcoal-stripped FBS. After 24 hours growth, various amounts of plasmid DNA [i.e., UASx5-Luc (400 ng), GAL4-NR4A1 (250 ng) and β-gal (250 ng)] were cotransfected into each well by GeneJuice Transfection reagent (Millipore Sigma, Darmstadt, Germany) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with plating media (as indicated above) containing either solvent (DMSO) or the indicated concentration of compound (in DMSO) for 18 hours. Cells were then lysed using a freeze–thaw protocol and 30 μL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against corresponding β- gal activity values as well as protein concentrations determined by Bradford assay.

5.2.6. Western blot analysis

Cells (3.0×10^5) were seeded on 6-well plate and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer RIPA (Thermo Scientific, Waltham, MA) that contained protease and phosphatase inhibitors

(GenDEPOT, Baker, TX). The total protein in the lysates was quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel; 35 μg of whole cell lysate were run in 8% of SDS page gels for G9a, mTOR and p-mTOR proteins and 25 μg of whole cell lysate were run in 10% of SDS page gels for the remaining proteins. The proteins from the gel were transferred to a PVDF membrane, then blocked for one hour using 5% skimmed milk. The membranes were incubated with primary antibody for 12 hours at 4 °C, then washed with Tris-buffered saline and Polysorbate 20 (TBST) and incubated with HRP-linked secondary antibody for 1 hour at 20 °C. The membranes were further washed with TBST and treated with Immobilon western chemiluminescence HRP-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA).

5.2.7. Transfection and small interfering RNAs.

For RNA interference experiment, cells were seeded on 6-well plates at 3×10^5/well then allowed 24 h to attach and grow. Then, they were transfected with siRNA of 100 nmol each/well for 6-well plates using 6.5 μl/well RNA iMax transfection reagent for 72 hours. siRNAs targeting NR4A1 (siNR4A1), Sp1 (siSp1), and Sp4 (siSp4) were purchased from Sigma-Aldrich. Negative Control Ig L2 siRNA were purchased from Qiagen. The oligonucleotides used were purchased from Millipore Sigma as follows:

siNR4A1_1, SASI_Hs02_00333289

siNR4A1_2, SASI_Hs02_00333290

siNR4A2: EHU008731

5.2.8. ChIP assay

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Cells (3×10^7) were treated with DMSO or indicated concertation of piperlongumine for 24 hours. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with 3 μg of normal IgG (abcam), NR4A1 (Abcam), Sp1 (Abcam), or RNA polymerase II (pol II; Abcam) antibodies and protein G-conjugated magnetic beads at 4 °C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. Reversed cross-link DNA was prepared by proteinase K digestion followed by Chromatin IP DNA purification (Active Motif). Then purified DNA products were analyzed by quantitative real-time PCR using amfiSure qGreen Q-PCR master mix (genDEPOT) using the manufacturer's protocol. The primers for detection of the G9a promoter region were F: 5-CAGATGGGGACAGAGACGC-3, R: 5-CCCGGAGCA TTGCACG-3. The primers for detection of the β1 integrin promoter region were F: 5-TCA CCA CCC TTC GTG ACA C-3(sense) and R: 5-GAG ATC CTG CAT CTC GGA AG-3(antisense).

5.2.9. Statistical analysis

Each assay was performed in triplicate and the results were presented as means with standard deviation (SD). The statistical significance of differences between the treatment groups was determined by Dunnett's multiple comparison test in ordinary one-way ANOVA. Gel analysis of Western Blotting was done using ImageJ (1.53K) software (RRID:SCR_003070). GraphPad Prism 8 (Version 8.4.3) software (RRID:SCR_002798) was used for analysis of variance and determine statistical significance. Data with a P value of less than 0.05 were considered statistically significant and indicated (*) in the figures.

5.3. Results

The 3,5-disubstituted DIM compounds (DIM-3,5) are potent anticancer agents using a breast cancer mouse xenograft model [274] and since there is a high degree of similarity between the LBDs of NR4A1 and NR4A2, we hypothesized that these compounds may be dual NR4A1/NR4A2 ligands. Figures 5-1A and 5-1B illustrate the binding of DIM-3,5-Cl_2 to the ligand binding domain of NR4A1 and NR4A2 respectively using direct binding (loss of fluorescence) and ITC assays. This compound binds both receptors and K^D values obtained for binding NR4A1 using direct binding and ITC assays were 7.7 and 0.001 µM respectively and the corresponding values for NR4A2 were 12.0 and 0.070 µM respectively (Figure 5-1A and 5-1B). Modeling studies (Fig. 5-1C) using Maestro-Schrodinger show that DIM-3,5-Cl_2 interacts with several amino acids in the LBD of NR4A1 and NR4A2 with docking scores of – 5.57 and – 4.37. These results demonstrate

that DIM-3,5-Cl_2 is a dual NR4A1/2 receptor ligand in which K_D values indicate higher binding affinities for NR4A1 compared to NR4A2.

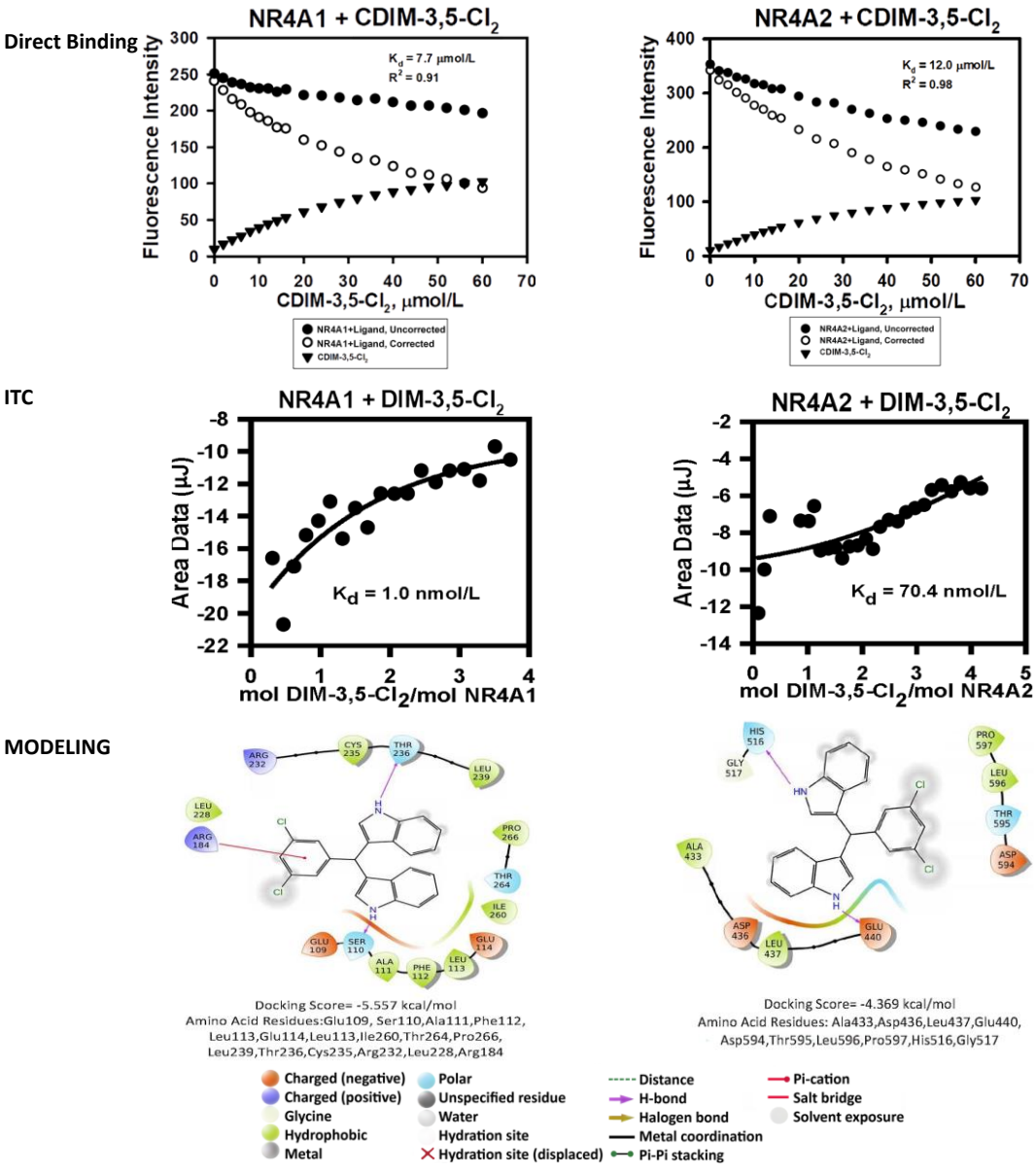


Figure 5 Error! No text of specified style in document.**-1. Interaction of DIM-3,5-Cl_2 with NR4A1 and NR4A2. DIM-3,5-Cl_2 was incubated with the LBD of NR4A1 or NR4A2 and binding curves and K_D values were determined using direct binding (loss of fluorescence) (A) or ITC (B) assays as outlined in the Methods section. C) Modeling**

of the interactions of DIM-3,5-CI_2 within the NR4A1 and NR4A2 binding sites was determined using Maestro/Schrodinger program as outlined in the Methods.

We also investigated comparable interactions of DIM-3,5-Br_2 (Figure 5-2) and DIM-3-Cl-5-CF3 (Figure 5-3) with the LBDs of NR4A1 and NR4A2 proteins. K_D values for interactions of DIM-3,5-Br_2 with NR4A1 and NR4A2 using the direct binding (Fig. 5-2A) and ITC (Fig. 5-2B) assays were 6.5 and 12.2 µM, and 0.042 and 0.020 µM respectively. Figure 2C illustrates the interactions of DIM-3,5-Br2 with amino acids in the LBDs of NR4A1 and NR4A2 and docking scores for binding NR4A1 and NR4A2 were – 4.00 and – 2.76. Figure 5-3 illustrates the binding of DIM-3-Cl-5-CF3 with the LBDs of NR4A1 and NR4A2 using the direct binding (Fig. 5-3A) and ITC (Fig. 5-3B) assays, and KD values for NR4A1 and NR4A2 were 3.1 and 5.5 µM, and 0.007 and 0.359 µM respectively. Figure 5-3C illustrates interaction of DIM-3-Cl-5-CF3 with the LBDs of NR4A1 and NR4A2 with docking scores of – 4.88 and – 4.2 respectively. Ligand binding to NR4A1 identified TMY301 and TMY302 as biding sites [158] and the docking scores for the TMY301-DIM-3,5 interactions were higher, and these values are illustrated in Figures 5-1 to 5-3. Modeling studies also showed 2 binding sites in the LBD of NR4A2 [396] and docking scores for DIM-3,5 compounds were higher for the site on chain B and these are also given in Figures 5-1 to 5-3. Interestingly, for NR4A2 modeling a comparison of amino acid side chain interactions for DIM-3,5-Cl_2, DIM-3,5-Br_2 and DIM-3-Cl-5-CF3 were similar: for NR4A1-DIM-3,5- interactions with amino acids side chains were similar for DIM-3,5-Br2 and DIM-3,5-Cl_2 whereas there were differences in amino acid interactions for DIM-3-Cl-5-CF3.

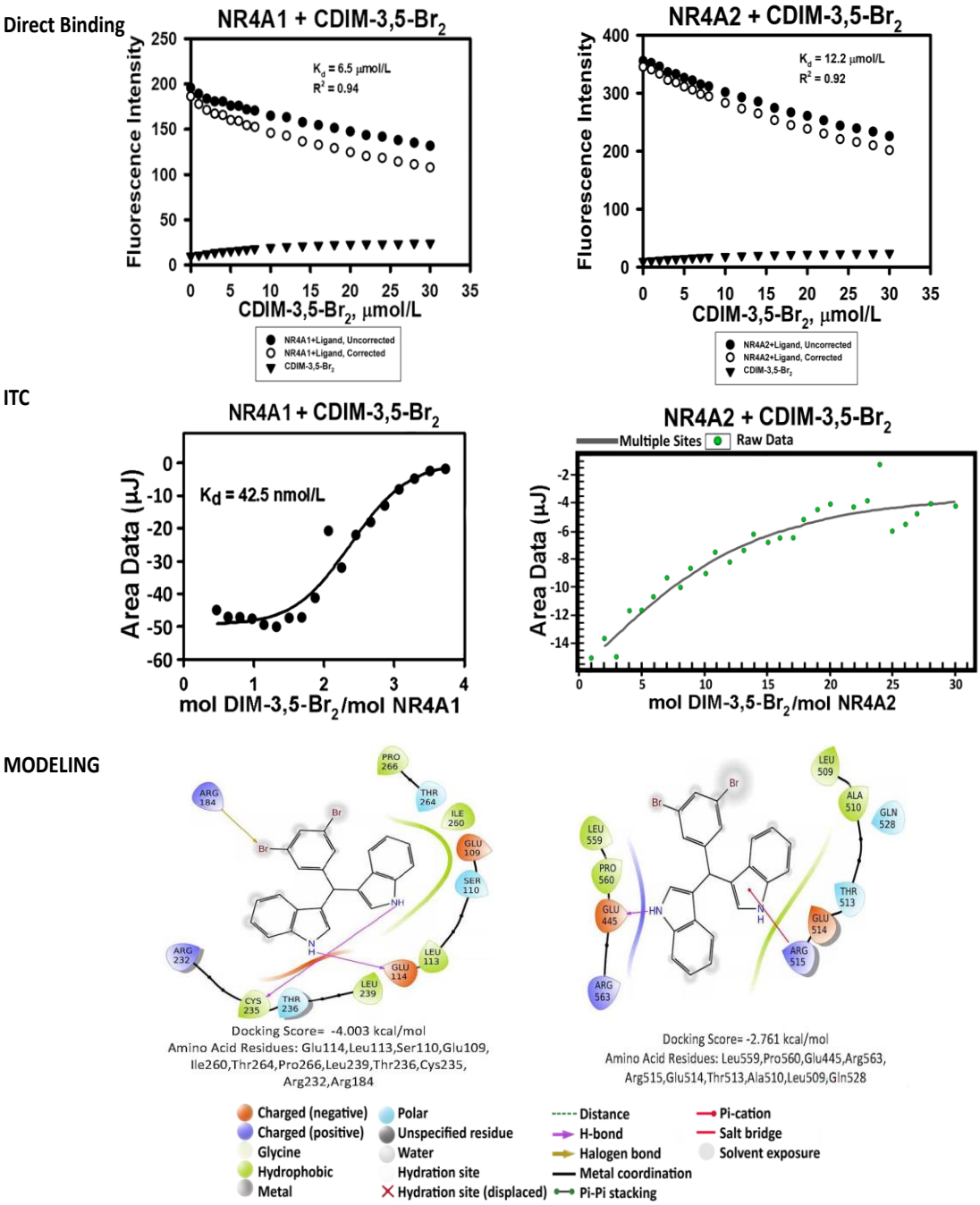


Figure 5-2. Interaction of DIM-3,5-Br_2 with NR4A1 and NR4A2. DIM-3,5-Cl_2 was incubated with the LBD of NR4A1 or NR4A2 and binding curves and KD values were determined using direct binding (loss of fluorescence) (A) or ITC (B) assays as outlined in the Methods section. C) Modeling of the interactions of DIM-3,5-Br_2 within the NR4A1 and NR4A2 binding sites was determined using Maestro/Schrodinger program as outlined in the Methods.

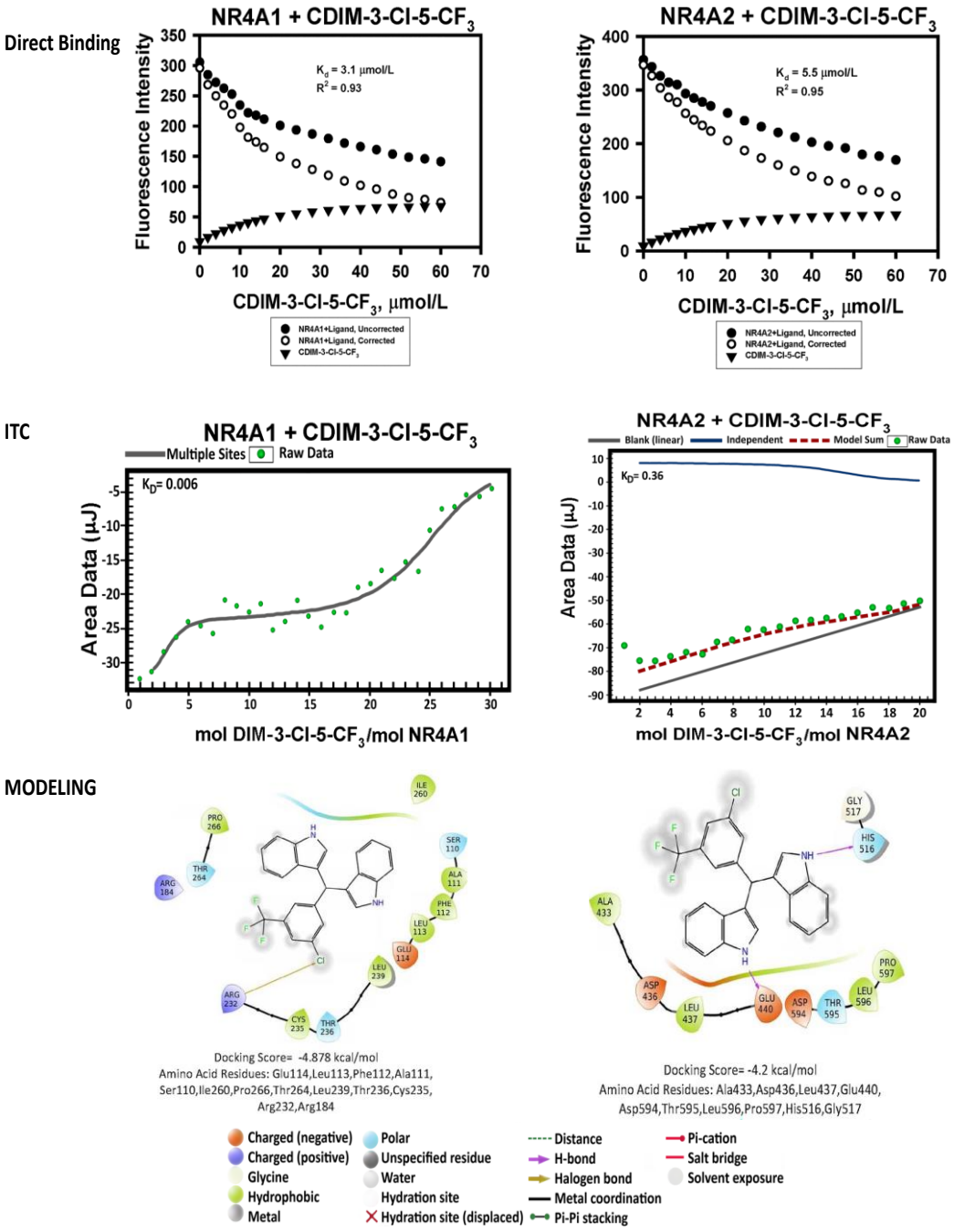


Figure 5-3. Interaction of DIM-3-CI-5-CF3 with NR4A1 and NR4A2. DIM-3-CI-5-CF3 was incubated with the LBD of NR4A1 or NR4A2 and binding curves and KD values were determined using direct binding (loss of fluorescence) (A) or ITC (B) assays as outlined in the Methods section. C) Modeling of the interactions of DIM-3-CI-5-CF3 within the NR4A1 and NR4A2 binding sites was determined using Maestro/Schrodinger program as outlined in the Methods.

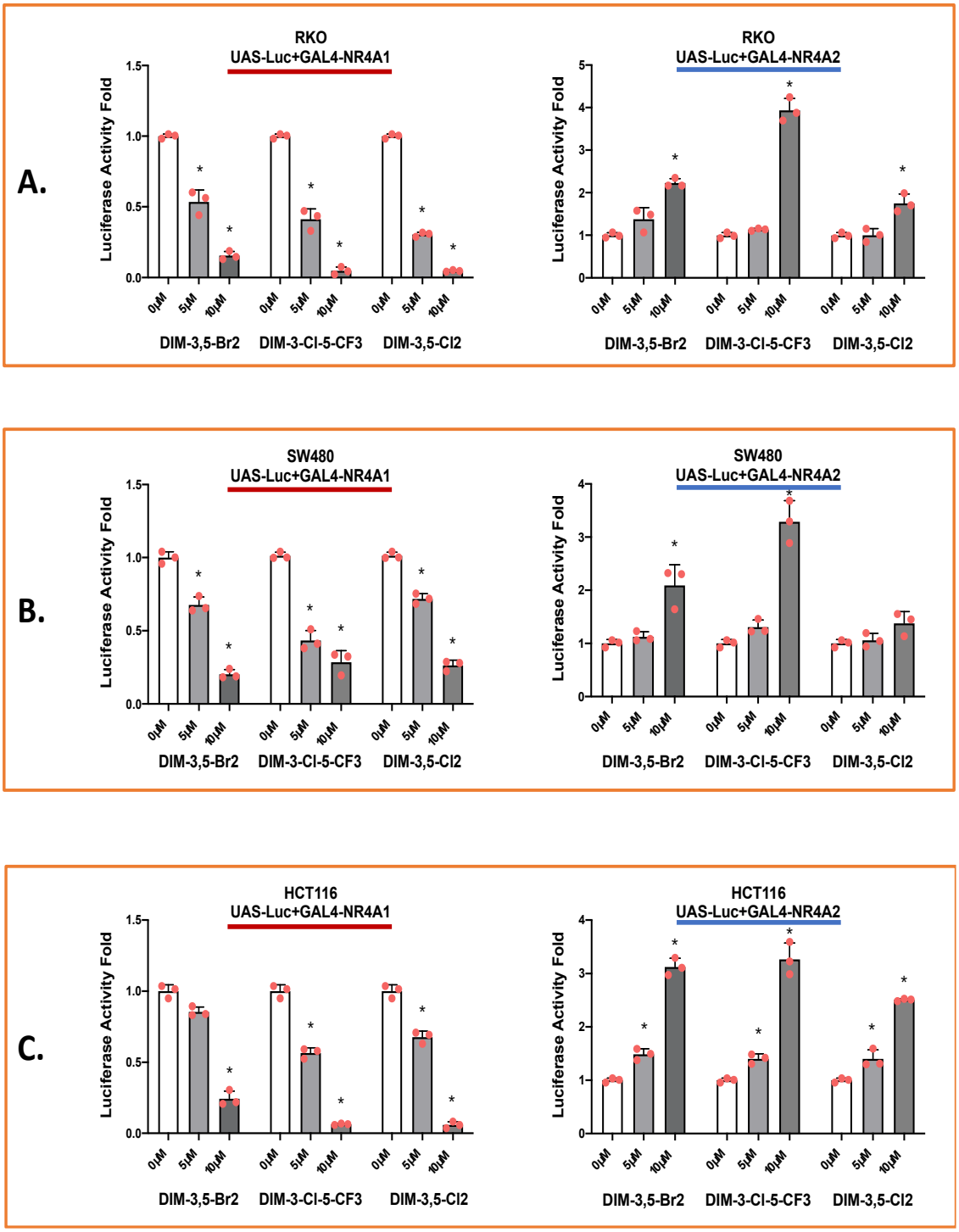


Figure 5-4. Effects of dual NR4A1/2 ligands on transactivation. RKO (A), SW480 (B) and HCT116 (C) cells were transfected with GAL4-NR4A1 or GAL4-NR4A2 and a UAS-luc reported gene, treated with DIM-3,5 analogs for 24 hours in the Methods. Results are expressed as means ± SD for at least 3 replicate determinations per treatment group and significant ($p < 0.05$) effects are indicated (*).

We also investigated effects of the dual NR4A1/2 ligands on transactivation in colon cancer cells transfected with a GAL4-NR4A1 or GAL4-NR4A2 (Fig. 5-4) chimera and a UAS-luc reporter gene containing 5 GAL4 response elements linked to a luciferase reporter gene. The dual receptor ligands significantly decreased NR4A1-dependent luciferase activity in RKO, SW480 and HCT116 cells and this response has previously been observed in other cancer cell lines with the CDIM/NR4A1 compounds [72, 157, 274]. In contrast, the dual NR4A1/2 compound increased NR4A2-dependent transactivation in colon cancer cells and the magnitude of these responses varied with only minimum induction observed for DIM-3,5-Cl2 in SW480 cells. Previous studies with the NR4A2 ligand, 1,1-bis(3'-indolyl)-1-(p-chlorophenyl) methane (DIM12) showed that this compound inhibits growth, migration and survival in pancreatic cancer cells but induces NR4A2-dependent transactivation [187, 190] as observed for the dual NR4A1/2 ligands in this study. Interestingly, the effects of NR4A2 ligands in functional vs transactivation assays in colon and pancreatic cancer cells is not observed in glioblastoma cells where NR4A2 ligands decrease growth and migration and also decrease NR4A2-dependent transactivation [191]. We also further investigated the effects of the 3 dual NR4A1/2 ligands as inhibitors of cell proliferation of RKO, SW480 and HCT 116 colon cancer cells (Figure 5-5). All 3 compounds significantly inhibited cell growth with IC50 values at approximately 10 μM and this is consistent with the effects of previous studies in cancer cell lines with CDIM compounds that were characterized as NR4A1 or NR4A2 ligands [191, 283].

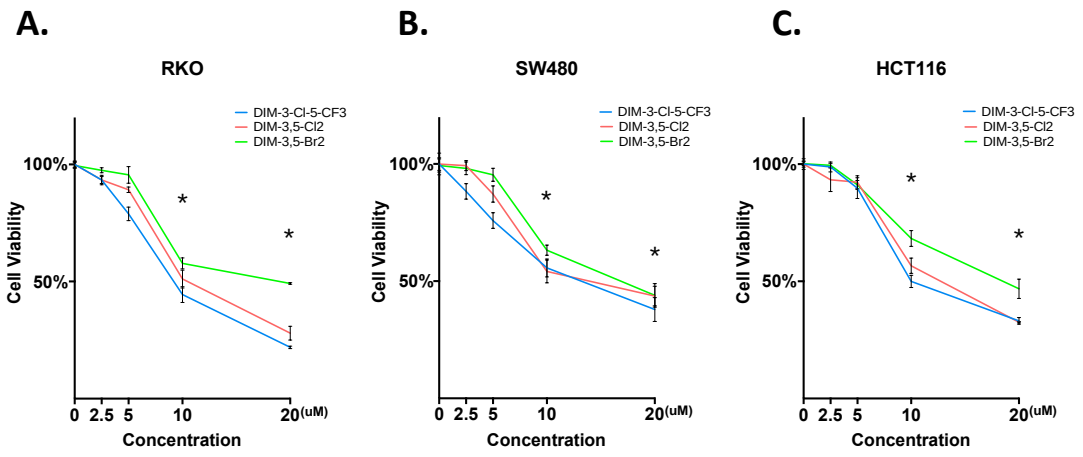


Figure 5-5. Effects of dual NR4A1/2 ligands on cell proliferation. RKO (A), SW480 (B) and HCT116 (C) cells were treated with DIM-3,5- compounds for 24 hours and cell proliferation was determined in an XTT assay as outlined in the Methods. Results are means ± SD for at least 3 replicate determinations and significant ($p < 0.05$) growth inhibition is indicated (*).

Previous studies show that both β1-integrin and G9a are regulated by NR4A1/Sp1 interacting with GC-rich promoter sequences where NR4A1 act as a cofactor of Sp1 [167, 281] and this is commonly observed for other nuclear receptors [86]. Figure 5-6A and 5-6B summarizes the effects of DIM-3,5-Cl_2 on interaction of NR4A1, NR4A2, Sp1 and Sp4 to the GC-rich regions of the β1-integrin and G9a regions of their promoters respectively. In DMSO treated cells NR4A1, NR4A2, Sp1 and Sp4 were associated with the β1-integrin and G9a promoters and treatment with the dual NR4A1/2 inverse agonist decreased association of both NR4A1 and NR4A2, Sp1 and Sp4 (only G9a) with these promoters. Sp4 exhibited slightly increased binding to the β1-integrin promoter. Thus, NR4A1 and NR4A2 act as cofactors of Sp in regulating expression of β1-integrin and G9a; it is not possible to distinguish if they act as monomers or dimers, and this could include an NR4A1-NR4A2 heterodimer since previous studies report interactions of these receptors [81].

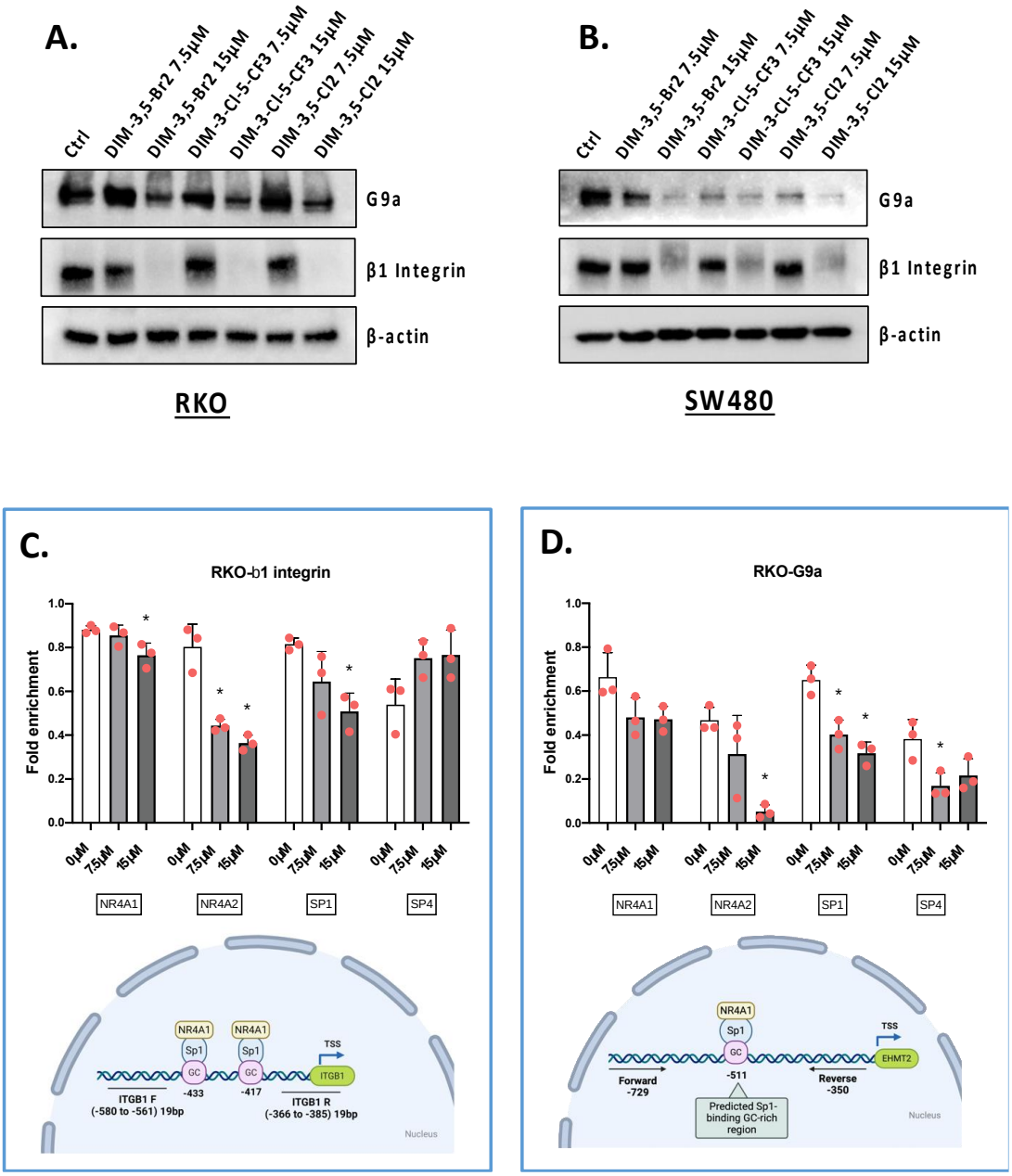


Figure 5-6. Dual NR4A1/2 ligands regulate expression of G9a and β1-integrin. Cells were treated with DIM-3,5 ligands for 24 hours and effects on β1-integrin (A) and G9a (B) protein levels were determined by Western blot analysis as outlined in the Method. RKO cells were treated with DIM-3,5-Cl2 for 24 hours and ChIP assays targeting the GC-rich regions of the β1-integrin (C) and G9a (D) promoters were used to determine association of NR4A1, NR4A2, Sp1 and Sp4 with the promoter regions using Q-PCR and outlined in the Methods section. The procedure was repeated in triplicate and results of PCR are presented as means ± SD; significant ($p < 0.05$) changes are indicated (*).

5.4. Discussion

NR4A family members NR4A1, NR4A2 and NR4A3 are orphan NRs for which endogenous ligands have not yet been identified [51]. These receptors are immediate early genes which are induced by diverse stressors, inflammation and one or more of NR4A are upregulated in diseases of inflammation and cancer [129, 367]. Based on the absence of endogenous ligands, NR4A are generally classified as ligand-independent transcription factors and this has been confirmed in multiple studies which have examined the effects of gene knockdown of specific phenotypes and genotypes. For example, in solid tumors knockdown of NR4A1 or NR4A2 results in decreased cancer cell proliferation, migration/invasion and induction of apoptosis. In studies on NR4A1 [72] several genes associated with these responses have been identified and these include downregulation of survivin, bcl2, Myc, phosphor-mTOR, epidermal growth factor receptor (EGFR), other receptor tyrosine kinases and several integrins and induction of apoptosis and apoptotic genes including cleared caspases and PARP. There is also evidence that some of the same genes are affected by NR4A2 ligands or NR4A1 knockdown including induction of apoptosis and enhanced caspase and PARP cleavage, decreased phosphor-mTOR, bcl2, Myc, and RTKs [191, 397-399].

Recent studies in this laboratory have synthesized and characterized a “third generation” set of CDIMs which contain a 3,5-disubstitutedphenyl moiety bonded to the methane carbon of 1,1-bi(3’indolyl) methane (DIM) [274]. Initial studies showed that these compounds bound NR4A1 in vitro and were highly potent inhibitors of breast tumor growth in an orthotopic athymic nude mouse model bearing triple negative MDA-MB-

231 cells as xenografts. All compounds tested inhibit tumor growth at doses of 1 mg/kg/day without any observable toxicity [274]. These results, coupled with observations that both NR4A1 and NR4A2 were primarily pro-oncogenic in solid tumors and that the LBDs of both receptors exhibited > 60% similarity led us to hypothesize that DIM-3,5 compounds may be dual receptor ligands and bind both NR4A1 and NR4A2. Results in Figures 1-3 demonstrate the 3 DIM-3,5 compounds, namely DIM-3,5-Cl2, DIM-3,5-Br2, and DIM-3-Cl-5-CF3 bind both NR4A1 and NR4A2 in a direct binding (loss of Trp fluorescence) and ITC assays. The ITC assay gave lower KD values for these compounds compared to the direct binding assay and this may be due to interactions of the DIM-3,5 compounds with the binding pocket and other sites in the LBD. Although there were some differences observed in the modeling of DIM-3-Cl-5-CF3 interaction with the LBD of NR4A1 the results showed that all 3 compounds bound with higher affinity (lower KDs) to the LBD of NR4A1 than NR4A2 in both assays.

Thus DIM-3,5-Cl2, DIM-3,5-Br2, and DIM-3-Cl-5-CF3 are dual NR4A1/2 ligands that decrease and increase NR4A1- and NR4A2-dependent transactivation in a GAL4-NR4A/UAS-luc assay in colon cancer cells and they also inhibit cell proliferation. Similar results were observed after knockdown of NR4A1 and NR4A2 on colon cancer cell apoptosis are ongoing. Previous studies showed that several genes are regulated by NR4A1/Sp1 a NR4A1/Sp4 in cancer cell lines where NR4A1 acts as an obligatory cofactor of Sp1 or Sp4 bound to GC-rich promoter sites [72, 84, 170, 283]. This is commonly observed for other NRs and after a screening study by RNA interference, we

observed that two previously NR4A1/Sp1 regulated genes, G9a and β1-integrin are also downregulated by DIM-3,5 in RKO and SW480 cells. We therefore used a ChIP assay and primers that encompass the GC-rich region of the β1-integrin and G9a genes and examined interactions of NR4A1, NR4A2, Sp1 and Sp4 with these sites by qPCR. The results indicate that all 4 transcription factors are associated with these sites in untreated RKO cells and after treatment with DIM-3,5-Cl2 there is a concentration-dependent loss of these interactions except for Sp4 which is increased on the β1-integrin promoter. These data are comparable to previous ChIP analysis of NR4A1, Sp1 and Sp4 interaction on other NR4A1/Sp1/4- regulated genes [84, 281, 283]. It is possible that NR4A1 and NR4A2 act independently or coordinately since previous studies show that these receptors interact [81] and this is currently being further investigated.

In summary, these studies demonstrate that DIM-3,5-Cl2, DIM-3,5-Br2, and DIM-3-C-5-CF3 are dual NR4A1/2 ligands that act as inverse NR4A1 and NR4A2 agonists in colon cancer cells. It has previously been observed that both prostaglandin A2 and celastrol are also dual NR4A1/NR4A2 ligands. Currently we are also investigating other anticancer compounds as dual receptor ligands, their relative KD values for NR4A1 and NR4A2 and potential for clinical applications where both receptors play a key role.

CHAPTER VI

CONCLUSIONS

Nuclear receptor 4A family (NR4As) are early immediate genes that play an important role in maintaining cellular homeostasis and are associated with a variety of diseases, including solid tumors, fibrosis, cardiovascular disease, neuronal disease, and metabolic disorders. The elevated expression of NR4A1/2 in these diseases suggests their potential as therapeutic targets. Inverse agonists of NR4A1/2, such as bis-indole-derived ligands, have shown promising results in preclinical studies, highlighting their potential to alleviate symptoms and improve outcomes in various diseases. The inverse agonist activity of these ligands inhibits NR4A1/2-dependent oncogenes and pathways, providing new avenues for disease treatment. In addition, natural products such as resveratrol and piperlongumine, and flavonoids such as quercetin and kaempferol have been identified as NR4A1 ligands in this study exhibit similar effects to inverse NR4A1 agonists. The results of this study suggest that the repurposing of natural products that are NR4A1 ligands may provide new therapeutic strategies and targets for diseases involving NR4A1.

Resveratrol is a polyphenolic compound whose chemotherapeutic properties have been extensively studied in preclinical models. Resveratrol levels are high in fruits, nuts and vegetables and has been associsted with many health benefits, including extending lifespan and preventing aging-related and inflammatory diseases, including cancer. However, the effectiveness of resveratrol in human clinical trials did not match the

promising results observed in preclinical studies. One possible reason for this difference is the poor bioavailability of resveratrol and its potential side effects, especially in certain cancers. This study has identified resveratrol as an NR4A1 ligand, binding to NR4A1 with high affinity and acting as an antagonist. Inhibitory effects of resveratrol on NR4A1-dependent transactivation in lung cancer cells have been demonstrated, suggesting that its anticancer activity may be mediated, at least in part, through NR4A1 inhibition. Furthermore, resveratrol exhibited similar effects to NR4A1 inverse agonists in regulating NR4A1-dependent genes and pathways, suggesting its potential as a selective receptor modulator. While the exact mechanism by which resveratrol interacts with NR4A1 and its impact on cancer therapy remains to be fully investigated, understanding these interactions may lead to the development of more targeted and precise treatments.

Piperlongumine, like other inverse NR4A1 agonists, inhibits cell growth, induces apoptosis, inhibits migration and invasion, acts as an mTOR inhibitor, induces ROS, and modulates expression of NR4A1-regulated genes has been investigated in this study. The selectivity of piperlongumine and other NR4A1 ligands may be attributed to their interactions with specific amino acid side chains within the NR4A1 ligand-binding domain (LBD). This study concludes that piperlongumine's anticancer activity is partly due to its inactivation of NR4A1, and further investigations are ongoing to explore its effects in other diseases where NR4A1 is a therapeutic target.

Endometriosis is a complex gynecological disorder characterized by the growth of endometrial tissue outside the uterus and NR4A1 is considered a promising target for the treatment of this disease. In endometriotic tissue, elevated levels of the nuclear receptor NR4A1 involved in various cellular processes including cell division, inflammation, programmed cell death, and fibrosis, all of which led to endometrial Atopic disease progression. To address the need for non-hormonal therapies with fewer side effects than current hormonal treatments, researchers have explored the use of NR4A1-targeting compounds. Diindole-derived compounds (CDIMs) have been identified as potent NR4A1 antagonists that inhibit the growth of human endometrial cells from patients with endometriosis. These CDIMs induced apoptosis (programmed cell death) and inhibited fibrotic pathways and inhibited growth of endometriotic lesions in a mouse model of endometriosis. In addition to synthetic NR4A1 ligands, natural products have also attracted attention for their therapeutic efficacy and relatively low toxicity. Flavonoids, a class of polyphenolic compounds found in various fruits and vegetables, have been identified as NR4A1 ligands that regulate NR4A1-mediated cellular pathways. For example, flavonoids such as kaempferol and quercetin inhibit growth of rhabdomyosarcoma cells by inhibiting NR4A1-regulated oncogenes and pathways. Based on these findings, we investigated the potential of flavonoids for the treatment of endometriosis. Several structurally diverse flavonoids, including quercetin, luteolin, myricetin, chrysin, and epigallocatechin-3-gallate, have demonstrated inhibitory effects on endometriotic cell growth, migration, and invasion in both in vitro and in vivo models of endometriosis. These natural compounds have shown promise in suppressing

endometriosis progression. The ability of flavonoids to modulate NR4A1-mediated pathways provides a mechanistic basis for their therapeutic effects observed in endometriosis. By targeting NR4A1, flavonoids can disrupt the abnormal cellular processes underlying endometriosis, such as aberrant cell growth, inflammation, and fibrosis. Moreover, these compounds offer the advantage of being derived from natural sources, making them attractive candidates for potential drug development due to their perceived safety and tolerability.

NR4A2 also plays a pro-oncogenic in cancer as observed for NR4A1 and inhibits cancer cell growth, migration/invasion, and induction of apoptosis. NR4A1/2 represent important targets for the treatment of various diseases, including cancer and endometriosis. Both synthetic compounds and natural products, such as CDIMs and flavonoids, have shown promise as NR4A1/2 ligands, exerting inhibitory effects on NR4A1/2-mediated pathways and demonstrating therapeutic potential. The ability of these compounds to modulate NR4A1/2 activity offers a novel approach for disease intervention, particularly in cases where NR4A1/2 is overexpressed or dysregulated. While further research is needed to fully understand the molecular mechanisms underlying the interactions between NR4A1/2 and its ligands, the current findings provide valuable insights for the development of targeted therapies. Future studies should focus on investigating the structure-dependent molecular interactions between NR4A1/2 and its ligands, as well as investigating their effects in a wider range of disease models and clinical settings. Additionally, optimizing the pharmacokinetic properties

and bioavailability of NR4A1/2 ligands, including improving their cellular uptake and reducing metabolism, could enhance their therapeutic efficacy. Overall, the identification and characterization of NR4A1/2 ligands offer a promising avenue for development of novel therapeutics for endometriosis and other diseases. Targeting NR4A1/2-mediated pathways has the potential to disrupt disease progression, inhibit abnormal cell growth, reduce inflammation, and attenuate fibrosis, thereby providing a multi-faceted approach for treatment. In addition to their direct effects on NR4A1/2, some compounds may also have indirect effects on other molecular targets or signaling pathways implicated in endometriosis. For instance, flavonoids not only act through NR4A1/2 but also modulate other signaling pathways, including the PI3K/Akt pathway, MAPK pathway, and NF-κB pathway, which are involved in cell survival, proliferation, and inflammation. By affecting these pathways, flavonoids may exert additional beneficial effects in the context of endometriosis. It is worth noting that while NR4A1/2-targeted therapies show promise, further research is needed to validate their efficacy, safety, and clinical utility. Preclinical studies using animal models have provided valuable insights, but the translation of these findings into future human clinical trials is necessary to determine the effectiveness of NR4A1/2-targeted interventions in patients with endometriosis. Rigorous evaluation of these compounds in terms of their pharmacokinetics, toxicity profiles, and potential drug interactions will be essential steps in the development process.

In summary, NR4As are promising therapeutic targets for cancer and endometriosis and hold promise for the development of innovative treatments. Both synthetic NR4A ligands, such as CDIMs, and natural compounds, have shown potential in modulating NR4A-mediated pathways and inhibiting disease progression. Continued research efforts focused on understanding the underlying molecular mechanisms, conducting rigorous preclinical and clinical studies, and optimizing therapeutic strategies will be crucial for harnessing the full therapeutic potential of using NR4A1/2 ligands for treating patients that overexpress these receptors.

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