NR4A orphan receptors and cancer

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NR4A orphan receptors are members of the nuclear receptor (NR) superfamily of transcription factors and include NR4A1 (Nur77, TR3, NGFI-B), NR4A2 (Nur1), and NR4A3 (Nor1). NR4A receptors are immediate-early genes induced by multiple stimuli and modulate gene expression by interacting as monomers or homodimers to NGFI-B response elements (NBREs) and Nur-responsive elements (NuREs), respectively. NR4A1 and NR4A2 (but not NR4A3) also form heterodimers with the retinoic acid X receptor (RXR) that bind a DRE motif, and there is evidence that NR4A1 can activate or deactivate gene expression in cancer cells through interactions with DNA-bound specificity protein 1 (Sp1) transcription factor. NR4A receptors play important roles in cellular homeostasis and disease, and there is increasing evidence that they exhibit pro-oncogenic activity in most tumors and thereby represent novel targets for chemotherapeutic drugs. Many apoptosis-inducing drugs induce nuclear export of NR4A1 and activate apoptosis in cancer cell lines through formation of extranuclear complexes including a pro-apoptotic mitochondrial NR4A1-bcl-2 complex. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methane analogs exhibit structure-dependent activation or deactivation of nuclear NR4A1 to induce apoptosis, whereas cytosporone B and structural analogs activate both nuclear and extranuclear NR4A1-dependent pro-apoptotic pathways. The roles of NR4A2 and NR4A3 in carcinogenesis are less well-defined; however, there is evidence suggesting that NR4A receptors are important targets for development of new mechanism-based anticancer drugs.

Introduction

The nuclear receptor (NR) superfamily of transcription factors regulates several key biological pathways in normal, diseased and neoplastic tissues, and new functions for these proteins are continually being discovered ([Bookout et al., 2006; Yang et al., 2006] and Bookout et al., unpublished results). There are 48 human NRs and, with few exceptions, these receptors exhibit a classical domain structure which includes an N-terminal (A/B) domain, a DNA-binding (C) and hinge region (D), and a C-terminal ligand binding (E and/or E/F) domain [Gronemeyer et al., 2004; McKenna et al., 2009].

Activation functions have been characterized in both the N- and C-terminal domains of most NRs and variants with deletions/mutations in several key domains have also been identified and investigated for their functions. Two receptors, namely small heterodimerization partner (SHP, NR0B2) and dosage-sensitive sex reversal, adrenal hyperplasia critical region on chromosome X, gene 1 (DAX1, NR0B1) do not have classical DNA binding domains and can only bind DNA in combination with a partner [Burr et al., 1996; Seol et al., 1996]. Twenty-five NRs have been classified as "orphan" receptors with no known endogenous ligands [Benoit et al., 2006; Shi, 2007]; however, this number has been decreasing as new ligands are identified. The molecular mechanisms associated with activation of NRs were initially derived from studies on the more classical ligand-activated receptors such as the estrogen receptor (ER). Each NR domain has specific functions and ligand binding within domain E results in receptor dimerization, conformational changes in the receptor, and interaction with cognate response elements. These events are accompanied by recruitment of coactivators and other nuclear coregulatory proteins which primarily interact with AF1 and AF2 in the N- and C-terminal domains, respectively [Hall and McDonnell, 2005; O'Malley, 2007; O'Malley and Kumar, 2009]. Selective receptor modulators (SRMs) exhibit tissue-specific agonist and antagonist activities for specific receptors and the activity of SRMs is complex and dependent on multiple factors including ligand structure, promoter and cell context, and expression of coactivator and other coregulatory proteins [Jordan and O'Malley, 2007; Katzenellenbogen et al., 1996; Smith and O'Malley, 2004]. The binding selectivity of various SRMs has resulted in the development of drugs such as tamoxifen and raloxifene that are extensively used for treatment of hormone-related diseases including breast cancer [Jordan, 2003; Jordan, 2009].
NR4A orphan receptors: structure and function

NR4A receptors: structure and DNA binding

The NR4A subfamily consists of three orphan receptors [Maxwell and Muscat, 2006; Pearen and Muscat, 2010], namely NR4A1 (Nur77, TR3, NGFI-B), NR4A2 (Nurr1), and NR4A3 (Nor1) that were initially identified as intermediate-early genes induced by nerve growth factor in PC12 cells [Milbrandt, 1988]. Figure 1 illustrates the domain structures of the three receptors; the well-conserved DNA binding and C-terminal ligand binding domains exhibit ~91-95% and ~60% homology, whereas their N-terminal A/B domains are highly divergent [Murphy et al., 1996; Paulsen et al., 1995; Saucedo-Cardenas et al., 1997].

Figure 1. NR4A receptors, their sequence homologies and domains. The major domains of human NR4A1, NR4A2 and NR4A3, their sequence homologies and number of amino acids are illustrated.

Although coactivators/coregulators have been linked to NR4A-mediated transactivation associated with both A/B (N-terminal) and E/F (C-terminal) domains [Castro et al., 1999; Chintharlapalli et al., 2005a; Maira et al., 2003; Ordentlich et al., 2003; Wansa et al., 2002; Wansa et al., 2003], the NR4A ligand binding domains (LBDs) are somewhat unique among NRs. The LBDs of NR4A receptors are more hydrophilic than other NRs and do not contain a hydrophobic cleft associated with the interactions of coactivators with steroid hormone receptors [Wansa et al., 2002; Wansa et al., 2003]. The X-ray crystal structure of NR4A2 (LBD) shows that the binding pocket of this receptor is occupied by bulky hydrophobic amino acid side-chains [Wang et al., 2003]. These results do not exclude the existence of endogenous or synthetic ligands, but also suggest that activation or deactivation of nuclear NR4A1, NR4A2 and NR4A3 may be ligand-independent, and due to posttranslational protein modifications such as phosphorylation, acetylation, ubiquitination and sumoylation which then facilitates recruitment of coactivators or possibly corepressors. This is consistent with results of studies showing that activation of NR4A receptors is dependent on several kinase pathways, including phosphatidylinositol-3-kinase (PI3K)/Akt, protein kinase A, and mitogen-activated protein kinases [Jacobs et al., 2004; Maira et al., 2003; Masuyama et al., 2001; Pekarsky et al., 2001; Stocco et al., 2002]. CR6-interacting factor 1 (CRIF-1) inhibits steroid receptor coactivator 2-mediated activation of NR4A1, in part, through inhibition of PKA activity [Park et al., 2005].

In common with other NRs, cognate DNA binding sites of NR4A receptors have been characterized and these include NGFI-B response element (NBRE) that binds monomers and the Nur-responsive element (NuRE) that binds homodimers and possibly heterodimers [Maira et al., 1999; Paulsen et al., 1995; Philips et al., 1997; Wilson et al., 1992; Wilson et al., 1991]. Moreover, NR4A1 and NR4A2 (but not NR4A3) form heterodimers with RXR that bind a DR5 motif [Perlmann and Janssön, 1995; Zetterstrom et al., 1996]. Activation or deactivation of genes by NR4A receptors has been investigated and the list of genes is extensive (reviewed in [Pearen and Muscat, 2010]); however, the identification of specific cis-elements is less well defined and the consensus NuRE has been characterized in a limited number of gene promoters including the pro-opiomelanocortin (POMC) gene in endocrine and lymphoid cells [Philips et al., 1997]. Research in this laboratory has reported a non-classical NR4A1-dependent activation of p21 and deactivation of survivin by methylene-substituted diindolylmethane derivatives (C-DIMs) in cancer cell lines and these involve NR4A1 interactions with specificity protein 1 (Sp1) transcription factor bound to GC-rich promoter elements [Lee et al., 2010; Lee et al., 2009]. It is likely that NR4A interaction with Sp proteins and other nuclear transcription factors may be an important mechanism for NR4A-dependent modulation of gene expression and these non-classical receptor-transcription factor (e.g., Sp1)-DNA complexes have also been observed for many other NRs including progesterone, androgen and estrogen receptors, retinoid receptors, peroxisome proliferator-activated receptor γ, COUP-TF and steroidogenic factor 1 [Liu and Simpson, 1999; Lu et al., 2000; Owen et al., 1998; Pipaon et al., 1999; Rohr et al., 1997; Safe and Kim, 2004; Safe and Kim, 2008; Shimada et al., 2001; Simmen et al., 1999; Sugawara et al., 2002; Sugawara et al., 2000; Suzuki et al., 1999].

NR4A receptors: endogenous physiological and pathophysiological functions

The identification of NR4A subgroup receptors as intermediate-early genes is consistent with the large number of stimuli that activate their tissue-specific expression. A recent review identified the following physiological and physical stimuli that induce expression of NR4A1, NR4A2 and NR4A3 and these include prostaglandins, fatty acids, growth factors, inflammatory cytokines, growth factors, neurotransmitters, calcium phorbol esters, hormonally-active peptides, and other cellular stressors including fluid shear stress, magnetic fields, and membrane depolarization [Maxwell and Muscat, 2006; Pearen and Muscat, 2010]. Many of these same agents and stressors also activate other immediate-early genes whose ultimate function is to maintain cellular homeostasis. Activation of NR4A receptors by multiple stressors is consistent with the increasing number of tissue-specific receptor-mediated effects that are important for their function. The extensive roles of NR4A1, NR4A2 and NR4A3 in metabolic processes, inflammation, vascular function, steroidogenesis, and the central nervous system have
NR4A knockout animal models have also provided insights into some of the endogenous functions of NR4A receptors; however, some redundancies among these receptors (particularly NR4A1 and NR4A3) may mask specific functions in mouse models [Fernandez et al., 2000; Murphy et al., 2001]. Although it was demonstrated that NR4A1 is required for T cell receptor-mediated apoptosis in T cells [Woronicz et al., 1994; Woronicz et al., 1995], NR4A1<sup>−/−</sup> mice appear to be normal with no apparent dysfunction in the thymus and this has been attributed to compensation by NR4A3 [Cheng et al., 1997; Lee et al., 1995]. NR4A2<sup>−/−</sup> mice die within 24 hr after birth and loss of this receptor results in severe neuronal deficits due to disruption of the dopaminergic system [Nsegbe et al., 2004], and studies with NR4A2<sup>−/+</sup> (heterozygotes) suggest that a reduction in dopamine levels and disruption of prepulse inhibition are linked to loss of this receptor [Eells et al., 2006]. Two different NR4A3<sup>−/−</sup> mice have been described; DeYoung and coworkers [DeYoung et al., 2003] reported that the homozygous null mice die at embryonic day 8.5 and that this receptor is crucial for gastrulation, whereas NR4A3<sup>−/+</sup> mice generated by Ponnio and coworkers [Ponnio et al., 2002] were viable and loss of this gene severely impacted the semicircular canals of the inner ear. Subsequent analysis of the “surviving” NR4A3<sup>−/−</sup> mice indicated loss of several functions including attenuation of neointima formation after vascular injury, decreased expression of adhesion molecules after inflammation, decreased hypocholesterolemia-induced atherosclerosis, and various neuronal deficits including enhanced susceptibility to seizures [Nomiyama et al., 2008; Ponnio and Conneely, 2004; Zhao et al., 2010]. It was also shown that NR4A3<sup>−/−</sup>NR4A1<sup>−/−</sup> double knockout mice rapidly developed lethal acute myeloid leukemia (AML) and died within 2-4 weeks after birth [Mullican et al., 2007]. Loss of both receptors was accompanied by dysregulation of multiple pathways and this included enhanced production of hematopoietic stem cells. These results clearly suggested a potential role for these receptors in leukemia and it was reported that both receptors were also decreased in leukemia patients [Mullican et al., 2007].

NR4A receptors and cancer

Expression of NR4A in cancer cells and patients

A systematic investigation of NR4A receptor levels in human tumors and in cancer cell lines has not been carried out; however, expression of these receptors (proteins) has been reported in multiple cancer cell lines [Ke et al., 2004; Li et al., 2006; Maruyama et al., 1995; Zhang, 2007]. Colon and pancreatic tumors express higher levels of NR4A1 in patient vs. control [Cho et al., 2007; Lee et al., 2010; Wu et al., 2010] and similar results have been observed in lung cancer patients (unpublished results). NR4A1 protein (cytoplasmic) was also overexpressed in breast cancer patients and there was an inverse correlation between NR4A1 expression with increasing histological grade of the tumor [Alexopoulou et al., 2010]. Cytoplasmic and nuclear NR4A2 protein expression was investigated in a cohort of bladder cancer patients and controls, and it was apparent that cytosolic and nuclear Nurr1 staining was higher in patients vs. controls and Nurr1 levels were associated with increasing histopathological grade of the tumor [Inamoto et al., 2010]. Although total Nurr1 protein expression was not a prognostic factor, higher levels of cytoplasmic Nurr1 correlated with higher tumor grade, distant metastasis and lower patient survival. Expression of NR4A1, NR4A2 and NR4A3 mRNAs has also been reported in publicly-available databases from β-cell lymphoma, breast, lung, and ovarian cancer patients [Lenz et al., 2008; Takeuchi et al., 2006; Yoshihara et al., 2010], and we have used these data for Kaplan-Meier survival analysis to determine possible correlations with receptor mRNA levels and patient survival. Using the median value to distinguish between high and low expression, there were no significant differences in survival between breast cancer and β-cell lymphoma patients and their NR4A1, NR4A2 or NR4A3 mRNA levels. Similar results were observed for lung cancer patients (using median values); however, examination of the top and bottom 30% of high and low NR4A1 expressors showed that lung cancer patients with high expression of NR4A1 exhibited longer survival than the low expression group (p<0.0126), whereas NR4A2 and NR4A3 mRNA were not prognostic factors (Figure 2). In the ovarian cancer cohort, there were differences observed using duplicate probes for each receptor and high expression of NR4A1 (p<0.0289) and NR4A2 (p<0.0203) correlated with increased patient survival. Microarray studies of mRNAs from cancer patients have identified a 17-gene signature associated with metastasis of solid tumors and downregulation of NR4A1 was part of this signature of metastasis [Ramaswamy et al., 2003]. It is apparent that NR4A1, NR4A2 and NR4A3 mRNA and protein expression is variable in patients with different types of tumors, and these receptors may have prognostic significance for patients with solid tumors.

Functional activity of NR4A receptors in cancer cells

The roles of NR4A receptors in cancer cell lines and tumors in terms of proliferation/anchorage-independent growth, survival/apoptosis, and migration/invasion (including angiogenesis) have not been well defined. However, a limited number of NR4A gene knockdown studies provide some insights into the function of these receptors in cancer cells. Table 1 summarizes some of the data obtained in various cancer cell lines and shows that knockdown of NR4A1 resulted in inhibition of cell growth, induction of apoptosis, and decreased angiogenesis, suggesting that NR4A1 is a pro-oncogenic factor in cancer [Bras et al., 2000; Ke et al., 2004; Kolluri et al., 2003; Lee et al., 2010; Wu et al., 2010; Zeng et al., 2006]. The only exception to these observations was observed in LNCaP cells, where antisense-mediated knockdown of NR4A1 appeared to increase cell growth [Uemura and Chang, 1998]. A recent paper reported that deoxycholic acid, a colon carcinogen, induced NR4A1 in...
mouse colon tissue (and cancer cells), suggesting that increased NR4A1 in colon tumors may be an important pro-oncogenic factor [Wu et al., 2010]. The effects of NR4A2 knockdown in bladder cancer cells did not affect cell proliferation, but decreased cell migration [Inamoto et al., 2010], and it was indicated in a review article [Li et al., 2006] that NR4A1, NR4A2 and NR4A3 were anti-apoptotic. A recent study investigated the effects of NR4A1 overexpression in mammary epithelial cells (226L and MCF10A cells) and breast cancer cell lines, and cell migration was decreased in 226L, MCF10A andZR75 (ER-positive), but not in the more highly aggressive MDA-MB-231 cells [Alexopoulou et al., 2010]. In contrast, overexpression of NR4A1 in lung cancer cells enhances proliferation and cell cycle progression [Kolluri et al., 2003]. These results demonstrate some differences between breast cancer cells vs. other cancer cell lines (Table 1), where NR4A knockdown studies suggest that NR4A1 and NR4A2 exhibit pro-oncogenic activity.

NR4A as a drug target - nuclear export pathways

The fascinating story of drug-induced nuclear translocation of NR4A1 and the resulting pro-apoptotic effects of extranuclear NR4A1 has been reviewed and these studies will only be briefly described [Zhang, 2007]. Despite the pro-oncogenic/anti-apoptotic effects of NR4A1 observed in most cancer cell lines, early in vitro and in vivo studies showed that overexpression of NR4A1 was associated with T cell receptor-mediated apoptosis in thymocytes [Liu et al., 1994; Wronicz et al., 1994]. Several reports have shown that the unusual caged retinoid, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437 or AHPN) induced apoptosis in lung and other cancer cell lines and this response was NR4A1-dependent [Cao et al., 2004; Dawson et al., 2001; Holmes et al., 2003a; Holmes et al., 2003b; Holmes et al., 2004; Kolluri et al., 2003; Li et al., 2000; Li et al., 1998; Lin et al., 2004b]. Interestingly, the NR4A1-dependent pro-apoptotic effects of CD437 were not due to the nuclear receptor, but rather to nuclear export of NR4A1, and studies on the NR4A1 domains required for this activity demonstrated that the DNA-binding domain was not required [Holmes et al., 2003b; Li et al., 2000; Lin et al., 2004b; Zhang, 2007]. Many other pro-apoptotic agents and stimuli are associated with nuclear export and/or induction of NR4A1 (Figure 3) and these include phorbol ester (TPA), butyrate, viruses, VP16, insulin-like growth factor binding protein 3 (IGFBP3), synthetic chenodeoxycholic acid derivatives, 5-fluourouracil, sulindac, HDAC inhibitors, calcium ionophores, cadmium, tin derivatives, cytosporone B and related analogs, n-butylenephthalide, and acetylsphikon derivatives [Chen et al., 2008b; Chinnaiyan et al., 2006; Gennari et al., 2002; Jeong et al., 2003; Lee et al., 2004; Lee et al., 2005; Liu et al., 2008; Liu et al., 2010; Liu et al., 2002; Shin et al., 2004; Wilson et al., 2003; Wu et al., 2002; Zhan et al., 2008].

The mechanisms associated with pro-apoptotic activity of NR4A1 have been extensively investigated and, even though NR4A1 does not contain prototypical mitochondrial target sequences, mitochondria are the major extranuclear targets of this receptor [Zhang, 2007]. Mitochondrial localization of NR4A1 and the subsequent release of cytochrome c from mitochondria are critical for the pro-apoptotic effects of this receptor [Li et al., 2000; Lin et al., 2004a], and it was discovered that activation of the intrinsic apoptotic pathway by NR4A1 was due to the formation of an NR4A1-bcl-2 pro-apoptotic complex [Lin et al., 2004a]. Moreover, an NR4A1-derived peptide that binds bcl-2 converts this survival factor into a pro-apoptotic complex, and the taxane anticancer drug Paclitaxel also binds NR4A1 as an "NR4A1 mimic" to activate apoptosis [Ferlini et al., 2009; Kolluri et al., 2008].
The unusual activity of a nuclear receptor acting as an extranuclear pro-apoptotic factor is dependent on intranuclear signals that induce nuclear export of NR4A1. NR4A1 can be phosphorylated at multiple sites, and using a chlorinated analog of the retinoid CD437, it was shown that activation of JNK was critical for drug-induced nuclear export of NR4A1, and this must also be accompanied by suppression of Akt [Han et al., 2006]. Akt-dependent phosphorylation of NR4A1 also blocks mitochondrial targeting of the receptor [Chen et al., 2008a]. However, in other cell lines, nuclear export of NR4A1 is dependent on different kinases [Katagiri et al., 2000] and it is likely that activation/deactivation of kinases required for nuclear export of NR4A1 may be complex and cell context-dependent. RXR forms a heterodimer with NR4A1 and many other orphan and adopted orphan NRs [Mangelsdorf and Evans, 1995; Tanaka and De Luca, 2009] and nuclear export of NR4A1 under some conditions also involves RXR and its ligands [Katagiri et al., 2000; Li et al., 2000; Lin et al., 2004b]. It should also be noted that butyrate-induced apoptosis in colon cancer cells was due to cytosolic and not mitochondrial targeting of NR4A1 [Wilson et al., 2003], and the mechanisms and contributions of this pathway require further investigation in colon and other cancer cells/tumors. It is clear that NR4A1 expression and drug-induced nuclear export are important elements in carcinogenesis and potential clinical application and the role of this pathway for other members of this receptor sub-family requires further investigation.

NR4A as a drug target: nuclear activation

Research in this laboratory has focused on a series of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methane (C-DIM) analogs (Figure 3) as anticancer agents and initial studies showed that several analogs inhibited carcinogen-induced mammary tumor development and growth in female Sprague-Dawley rats [Qin et al., 2004]. Since C-DIMs are small lipophilic molecules, their potential as NR agonists or antagonists was investigated in bioassays using GAL4-receptor chimeras containing a receptor fused to the yeast GAL-4 DNA-binding domain (aa 1-147) and a reporter gene (pGAL4-luc) containing 5 tandem GAL4 response elements linked to a luciferase reporter gene. Several C-DIMs including the p-trifluoromethyl (DIM-C-pPhCF3), p-t-butyl (DIM-C-pPhtBu), p-cyano (DIM-C-pPhCN), and p-phenyl (i.e., biphenyl) (DIM-C-pPhC6H5) activated peroxisome proliferator-activated receptor γ (PPARγ) [Qin et al., 2004]. These compounds inhibited breast [Qin et al., 2004; Su et al., 2007; Vanderlaag et al., 2008], cervical and endometrial [Hong et al., 2008], colon [Chintharlapalli et al., 2005b; Chintharlapalli et al., 2006; Chintharlapalli et al., 2004], bladder [Kassouf et al., 2006], ovarian [Lei et al., 2006], prostate [Chintharlapalli et al., 2007; Papineni et al., 2008], lung [Ichite et al., 2009], pancreatic [Guo et al., 2010], and kidney [York et al., 2007] cancer cells and tumor growth in xenograft and orthotopic models. However, it was also apparent that the anticancer activities of the PPARγ-active C-DIMs and other analogs were PPARγ-independent in most cancer cell lines. These

### Table 1. Functional effects of NR4A gene silencing in cancer cell lines

<table>
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<tr>
<th>Cell Line</th>
<th>Growth</th>
<th>Apoptosis / Survival</th>
<th>Invasion / Angiogenesis</th>
<th>Reference</th>
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<tr>
<td>NR4A1</td>
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<td>HeLa (cervical)</td>
<td></td>
<td>apoptosis ↑</td>
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<td>Ke et al. 2004</td>
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<tr>
<td>M14 (melanoma)</td>
<td></td>
<td>apoptosis ↑</td>
<td></td>
<td>Ke et al. 2004</td>
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<tr>
<td>B16 (melanoma)</td>
<td></td>
<td>apoptosis ↑</td>
<td>angiogenesis ↓</td>
<td>Zeng et al. 2006</td>
</tr>
<tr>
<td>A20B (lymphoma)</td>
<td></td>
<td>apoptosis ↑</td>
<td></td>
<td>Bras et al. 2000</td>
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<tr>
<td>H460 (lung)</td>
<td>inhibition</td>
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<td>Kolluri et al. 2003</td>
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<td>LNCaP (prostate)</td>
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<td>enhanced</td>
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<td>Uemura et al., 1998</td>
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<tr>
<td>HCT116 (colon)</td>
<td>inhibition (c)</td>
<td>apoptosis ↑</td>
<td></td>
<td>Wu et al. 2010</td>
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<tr>
<td>Panc1, L3.6pL, MiaPaCa2 (pancreatic)</td>
<td>inhibition</td>
<td>apoptosis ↑</td>
<td></td>
<td>Lee et al. 2010</td>
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<tr>
<td>NR4A2</td>
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<td>Inamoto et al. 2010</td>
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<tr>
<td>UM-UC13 (bladder)</td>
<td></td>
<td>no change</td>
<td>decreased</td>
<td>Inamoto et al. 2010</td>
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See text for details.
Figure 3. Compounds that activate NR4A1 and induce apoptosis in cancer cell lines. CD437, C-DIMs and CsnB activate or deactivate NR4A1 (Cao et al., 2004; Lee et al., 2009; Zhan et al., 2008); 6-MP activates NR4A2 and NR4A3 (Ordentlich et al., 2003; Yoo et al., 2007); substituted imidazoles activate NR4A2 (Dubois et al., 2006); and prostaglandin A2 activates NR4A3 (Kagaya et al., 2005).

Compounds induced endoplasmic reticulum (ER) stress and the extrinsic pathway of apoptosis [Abdelrahim et al., 2006; Lei et al., 2008a; Lei et al., 2008b] and also directly targeted the mitochondria and decreased mitochondrial membrane potential (MMP) [Hong et al., 2008].

Initial screening studies using GAL4-NR4A1/pGAL4-luc constructs identified DIM-C-pPhOCH$_3$ and p-methoxy (DIM-C-pPhOCH$_3$) and unsubstituted (DIM-C-Ph) analogs as activators of NR4A1 [Chintharlapalli et al., 2005a] in pancreatic cancer cells and this was also observed in colon cancer cells [Cho et al., 2007]. However, the GAL4-NR4A1 construct used in these studies was derived from mouse NR4A1 [Sohn et al., 2001] and we subsequently repeated the transactivation studies in Panc28 cells using a GAL4-NR4A1 (human) construct which was cloned and sequenced in this laboratory [Lee et al., 2010]. The results (Figure 4) show only modest induction of transactivation by DIM-C-pPhOCH$_3$ in cells transfected with GAL4-NR4A1 (human)/GAL4-luc (reporter) or an NBRE-luc construct, whereas in previous studies, induced transactivation was much higher.

Despite these differences in the transactivation studies using GAL4-NR4A1 (human vs. mouse) constructs, DIM-C-pPhOCH$_3$ induced apoptosis in pancreatic and colon cancer cell lines and these responses were partially inhibited in cells transfected with a small inhibitory RNA that knocked down NR4A1 (siNR4A1) [Chintharlapalli et al., 2005a; Cho et al., 2007]. DIM-C-pPhOCH$_3$ has also...
been used in combination with RNA interference to investigate the TR3-dependent pro-apoptotic responses induced by this compound [Chintharlapalli et al., 2005a; Cho et al., 2010; Cho et al., 2007; Lee et al., 2009].

Microarray studies in thymocytes undergoing NR4A1-dependent apoptosis identified FasL and TRAIL as two of several induced pro-apoptotic genes [Rajpal et al., 2003]. Initial studies showed that TRAIL was also induced by DIM-C-pPhOCH$_3$ in colon, pancreatic and bladder cancer cells [Chintharlapalli et al., 2005a; Cho et al., 2010; Cho et al., 2007], and a recent study also showed that FasL was induced in L3.6pL pancreatic cancer cells (unpublished results).

DIM-C-pPhOCH$_3$-induced TRAIL and FasL was decreased in pancreatic cancer cells transfected with siNR4A1 and the induction of apoptosis and pro-apoptotic genes by DIM-C-pPhOCH$_3$ was observed without nuclear translocation of NR4A1, as determined by immunostaining and western blots of nuclear extracts.

Moreover, DIM-C-pPhOCH$_3$-induced apoptosis was unaffected in cells cotreated with leptomycin B, which inhibits nuclear protein export. Therefore, NR4A1-dependent induction of apoptosis with C-DIMs contrasted to the effects of apoptosis-inducing agents such as TPA and CD437, which require nuclear export of NR4A1 to mitochondria [Zhang, 2007]. The unique pro-apoptotic effects of DIM-C-pPhOCH$_3$ include activation of nuclear TR3 and also receptor-independent induction of ER stress and DR5, which subsequently activates the extrinsic apoptotic pathway [Cho et al., 2008; Lei et al., 2008a; Lei et al., 2008b].

Microarrays have been used to investigate activation of gene expression by DIM-C-pPhOCH$_3$ in multiple cancer cell lines ([Cho et al., 2010; Cho et al., 2007] and unpublished results). Among the genes induced in colon, pancreatic, bladder and prostate cancer cell lines, there were considerable cell context-dependent differences in expression of individual genes. Nineteen genes were significantly induced or decreased after treatment of bladder, pancreatic, prostate or colon cancer cells with 15 μM DIM-C-pPhOCH$_3$ for 2 or 6 hr. Seven genes were decreased in the four cancer cell lines (9TRAF5, CCNE2, TRIP10, SEMA4D, FEN1, JAG2 and TREXI); seven genes were induced (CEBPβ, HMOX1, SESN2, LAMP3, GDF15, PP11R15A and GDF15); and four genes were induced/decreased (DNAJB9, TXN1P, RGS19 and RASSF5) in a cell context-dependent manner.

RNA interference studies (siNR4A1) were used to confirm induction of NR4A1-dependent genes by DIM-C-pPhOCH$_3$ in colon, bladder and pancreatic cancer cells. DIM-C-pPhOCH$_3$ induced TRAIL in all three cell lines and the induction response was decreased by siNR4A1. Cystathionase (CSE) was induced in all three cell lines, and three genes were induced in two of the three cancer cell lines, namely ATF3, p21 and GDF15 (NAG-1). Sestrin 2 (SESN2), programmed cell death gene 1 (PDCD1), p8 and dual specificity phosphatase 1 (DUSP1) were induced in only one of the three cancer cell lines. With the exception of GDF15, induction of the remaining genes by DIM-C-pPhOCH$_3$ was decreased after cotransfection with siNR4A1. The expression of PDCD1, CSE and ATF3 in RKO, SW480, HCT-116, HT-29 and HCT-15 colon cancer cells showed that the three genes were induced in RKO and HT-29 cells; however, their induction in the other three cell lines was highly variable and not coordinated [Cho et al., 2007]. Thus, both tumor type and cell context were important for activation of this gene set by DIM-C-pPhOCH$_3$. 

![Figure 4. DIM-C-pPhOCH$_3$-induced transactivation in pancreatic cancer cells. Panc1 cells were transfected with GAL4-NR4A1 (human)/pGAL4-luc or an NBRE-luc construct and treated with 15 μM DIM-C-pPhOCH$_3$, and luciferase activity was determined as previously described (Lee et al., 2010). Significant (p-value < 0.05) induction of activity as indicated (*).](image-url)
Figure 5. Cell context-dependent induction of NR4A1-dependent genes by DIM-C-pPhOCH$_3$ in colon cancer cell lines. Colon cancer cell lines were treated with 12.5 µM DIM-C-pPhOCH$_3$ for 6 hr and expression of ATF3, CSE and PDCD1 mRNAs were determined by real time PCR, as described (Cho et al., 2007).

DIM-C-pPhOCH$_3$-induced expression of p21 was used as a model for mechanistic studies [Lee et al., 2009]. DIM-C-pPhOCH$_3$ induced p21 mRNA and protein levels in pancreatic cancer cells and transfection with siNR4A1 blocked induction, whereas leptomycin D did not affect the induction response. DIM-C-pPhOCH$_3$ also induced transactivation in pancreatic cancer cells transfected with p21 promoter constructs that contain the proximal GC-rich region of the p21 promoter, but not an NBRE or NuRE. Results of RNA interference studies show the induction of p21 by DIM-C-pPhOCH$_3$ is dependent on NR4A1 and Sp1 or Sp4 (but not Sp3) and involves NR4A1/Sp1/Sp4 interactions at the proximal GC-rich region of the p21 promoter (Figure 6). This mechanism is comparable to the induction of p21 in pancreatic cancer cells by PPARγ-active C-DIMs, due to PPARγ/Sp1/Sp4 interactions with p21 promoter constructs containing the same proximal GC-rich elements [Hong et al., 2004].

The p-hydroxy C-DIM analog (DIM-C-pPhOH) did not activate NR4A1 in initial transactivation studies in Panc28 cells and inhibited growth (10-20 µM) only after prolonged incubation (>48 hr) [Chintharlapalli et al., 2005a]. In L3.6pL, MiaPaCa-2 and Panc1 cells, growth inhibition was observed after treatment for 24-48 hr [Lee et al., 2010]. DIM-C-pPhOH did not induce nuclear export of NR4A1 and partially inhibited DIM-C-pPhOCH$_3$-induced responses in Panc28 cells [Chintharlapalli et al., 2005a].

DIM-C-pPhOH inhibited basal p21 expression in pancreatic and colon cancer cells [Guo et al., 2010], whereas DIM-C-pPhOCH$_3$ and PPARγ-active C-DIMs induced p21 [Lee et al., 2009]. The effects of DIM-C-pPhOH on NR4A1-dependent transactivation was investigated in pancreatic cancer cells transfected with GAL4-NR4A1 (human) chimeras containing wild-type and N-terminal (A/B) and C-terminal (C-F) truncated mutants. DIM-C-pPhOH significantly decreased transactivation in cells transfected with GAL4-NR4A1(wt) and GAL4-NR4A1(A/B), whereas endogenous activity of GAL4-NR4A1(C-F) was low and unaffected by the compound [Lee et al., 2010].

Since RNA interference studies indicate that NR4A1 exhibits pro-oncogenic activity in most cancer cell lines (Table 1), we directly compared the effects of siNR4A1 and DIM-C-pPhOH in pancreatic cancer cell lines. Both siNR4A1 and DIM-C-pPhOH decreased cell proliferation, induced apoptosis (Annexin V staining), increased caspase 3 and PARP cleavage, and decreased both survivin and bcl-2 protein expression. Using survivin as a model, DIM-C-pPhOH and siNR4A1 also decreased luciferase activity in cells transfected with constructs [pGL-SVV(-269)/pGL3-SVV(-150)] containing the GC-rich proximal promoter region of the survivin promoter. Similar results were observed with constructs containing multiple (3) consensus GC-rich elements that bind Sp1 and other...
Figure 6. Mechanisms of NR4A1-dependent activation or deactivation of gene expression by C-DIMs. The proposed mechanisms are derived from studies on activation of p21 by DIM-C-pPhOCH3 (Lee et al., 2009) and deactivation of survivin expression (Lee et al., 2010) in pancreatic cancer cells.

Sp proteins; however, Sp1 levels were not affected by DIM-C-pPhOH or siNR4A1. The mechanism of DIM-C-pPhOH-mediated downregulation of survivin was further investigated and we hypothesized that since p300 coregulates expression of both NR4A1 and Sp1, then p300 may also coregulate NR4A1/Sp1-mediated expression of survivin. p300 knockdown by RNA interference decreased basal activation of the GC-rich survivin construct; survivin protein was decreased, and PARP cleavage was increased. Thus, basal expression of survivin in pancreatic cancer cells is dependent on Sp1, NR4A1 and p300. In ChIP assays, treatment with DIM-C-pPhOH dramatically decreases p300 interactions with the GC-rich region of the survivin promoter and this is accompanied by minimal or no loss of TR3 or Sp1 binding (Figure 6). Ongoing studies in several cancer cell lines suggest a similar mechanism of action for decreasing expression of other genes, and we are currently developing DIM-C-pPhOH and other NR4A1 inactivators for future clinical applications in cancer treatment.

Cytosporone B and related compounds as NR4A1 agonists

Cytosporone B (Csn-B) (Figure 3) appears to be a unique NR4A1 agonist which binds and activates the ligand-binding domain of NR4A1 and induces nuclear NR4A1-dependent gene expression and nuclear export of the receptor [Zhan et al., 2008]. Activation of nuclear NR4A1 was confirmed in multiple experiments showing that Csn-B induces NR4A1 expression through specific interactions with NBRE sites in the NR4A1 promoter. It was also demonstrated using fluorescence quenching experiments that Csn-B binds to NR4A1 (wild-type) and the ligand binding domain (354 to 598) of NR4A1, and these experiments established Csn-B as a unique activator of nuclear NR4A1. Moreover, Csn-B-induced nuclear export of the receptor results in NR4A1-mitochondrial protein interactions and induction of apoptosis, which are similar to the effects observed for apoptosis-inducing agents [Zhan et al., 2008]. Liu and coworkers [Liu et al., 2010] used Csn-B as a scaffold for the synthesis of other NR4A1 agonists and identified several potent analogs that exhibit comparable mechanisms of action, namely activation of nuclear NR4A1 coupled with export of nuclear NR4A1 to mitochondria. Both Csn-B and related analogs inhibit tumor growth in murine xenograft models and induce apoptosis in tumors. Interestingly, the relative contributions of the ligand-induced nuclear and extranuclear NR4A1-mediated responses may be ligand-dependent. NR4A1 is both nuclear and cytosolic in tumors from mice treated with Csn-B [Zhan et al., 2008], whereas in tumors from mice treated with the potent cytosporone B analog pentyl-2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate, NR4A1 was primarily in the cytosol [Liu et al., 2010]. Csn-B and related compounds are the first ligands identified that bind and activate nuclear NR4A1 and resemble NR4A1-active C-DIMs in their ability to induce nuclear NR4A1-dependent transactivation. Csn-B and analogs also resemble apoptosis-inducing agents that induce export of the receptor and induces NR4A1-dependent apoptosis through mitochondrial disruption (Figure 6). NR4A1-active C-DIMs also induce receptor-independent apoptosis through receptor-independent induction of ER stress [Lei et al., 2008a; Lei et al., 2008b].

NR4A2 (Nurr1) and NR4A3 (Nor1) agonists

6-Mercaptopurine (6-MP) (Figure 3) is an antiproliferative and cytotoxic anticancer agent and induces expression of NR4A receptors in cancer cell lines [Yoo et al., 2007]. 6-MP also activates NR4A2-dependent transactivation through the N-terminal A/B domain and structure-activity studies among several purine/pyrimidine analogs demonstrate the specificity of the induction process; however, 6-MP-induced activity is inhibited by other
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nucleotides (adenosine, inosine, guanosine) [Ordentlich et al., 2003]. Activation of NR4A2 by 6-MP was also inhibited by the adenosine kinase inhibitor 5-iodotubercidin, suggesting that an active metabolite of 6-MP may be required for activation of NR4A2. Several substituted benzimidazoles (Figure 3) have also been identified as NR4A2 agonists using a library screening/synthesis approach and their EC50 values for activating a NuRE in MN9D cells (derived from the ventral mesencephalon) were < 100 nM [Dubois et al., 2006]. Isoxazolo-pyridinones were also potent agonists using a similar assay [Hintermann et al., 2007]; however, their activity and function in cancer cell lines were not reported.

Initial studies in bladder cancer cells identified 1,1-bis(3’-indolyl)-1-(p-chlorophenyl)methane (DIM-C-pPhCl) as a specific activator of NR4A2 [Inamoto et al., 2008]. This compound induced apoptosis and inhibited bladder cancer cell and tumor (orthotopic) growth, and the induction of TRAIL and PARP cleavage by DIM-C-pPhCl in bladder cancer cells was partially inhibited by knockdown of NR4A2 by RNA interference. Ongoing studies in this laboratory have identified several NR4A2 agonists, and their mechanisms of action and antineoplastic activity are currently being investigated.

6-MP activates NR4A3 through the N-terminal domain [Wansa et al., 2003], and the effects are similar to those described for activation of NR4A2 [Ordentlich et al., 2003], even though there is minimal similarity in the A/B domains of NR4A2 and NR4A3. Prostaglandin A2 (Figure 3) also activates NR4A3, but through the ligand binding domain [Kagaya et al., 2005]; however, the effects of NR4A3 agonists in cancer cell lines have not been described.

Summary

NR4A receptors play an important role in cancer and represent a novel target for development of anticancer drugs. There is evidence in multiple tumor types that NR4A1 is a pro-oncogenic factor and, in colon cancer, NR4A1 may contribute to cancer development. Agents that activate NR4A1 and NR4A2 inhibit cancer cell and tumor growth through both nuclear and extranuclear pathways, and deactivation of NR4A1 also inhibits tumor growth and induces apoptosis in cancer cells and tumors. Figure 6 illustrates the proposed pathways associated with nuclear NR4A1-dependent induction of p21 by DIM-C-pPhOCH3 and repression of survivin by DIM-C-pPhOH, demonstrating that both activation and deactivation of nuclear NR4A1 lead to growth inhibition and apoptosis. The identification of cytosporone B and related analogs and C-DIM compounds as ligands/activators and deactivators of NR4A1 will lead to synthesis of more potent anticancer agents and should spur research on further development of NR4A1-, NR4A2- and NR4A3-active compounds for cancer chemotherapy.

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