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REVIEW

Classical and novel retinoids: their targets in cancer therapy

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Retinoids are important mediators of cellular growth and differentiation. Retinoids modulate the growth of both normal and malignant cells through their binding to retinoid nuclear receptors and their subsequent activation. While retinoids have demonstrated therapeutic efficacy in the treatment of acute promyelocytic leukemia, their spectrum of activity remains limited. Other agents such as histone deacetylase inhibitors may significantly increase retinoid activity in a number of malignant cell types. The novel retinoids N-(4-hydroxyphenyl) retinamide (4-HPR) and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437; AHPN) induce apoptosis in a wide variety of malignant cells. Their mechanism(s) of action remain unclear, although a number of potential targets have been identified. Whether the retinoid receptors are involved in 4-HPR and CD473/AHPN mediated apoptosis remains unclear. Both 4-HPR and CD437/AHPN display significant potential as therapeutic agents in the treatment of a number of premalignant and malignant conditions.
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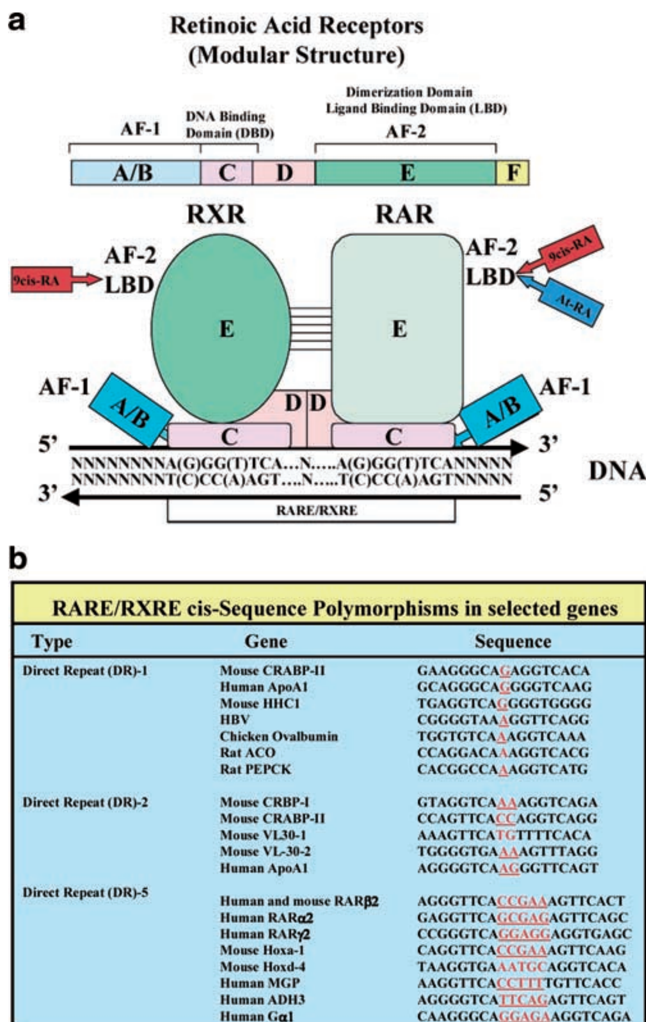
Introduction

The ability of retinoids to modulate a variety of important cellular functions has been well documented.[1,2] These agents have displayed therapeutic activity against a number of proliferative diseases but display a very limited role in the treatment of malignancies.[3–6] Recent developments in the synthesis of novel retinoids some of which possess unique mechanism(s) of action has resulted in broader spectrum of activity of these compounds, as well as a heightened enthusiasm for their potential role in the treatment of malignancies.

Retinoid receptors

Retinoids exert their effects through their ability to bind to and activate a number of nuclear receptors which function as transcriptional factors and, in turn, regulate the expression of the retinoid target genes.[7] The retinoid nuclear receptors are members of the steroid/thyroid hormone family of receptors. Two classes of retinoid receptors have been identified, ie the retinoic acid receptors (RARs) and the retinoid X repeaters (RXRs).[8] RARs are activated by both *trans* retinoic acid (RA) and 9-*cis*-retinoic acid (9-*cis*-RA), while the RXRs are activated only by 9-*cis*-RA.[9,10] Three subtypes, designated α, β and γ exist for each class.[11–13] Each receptor subtype has also been demonstrated to have multiple isoforms due to differential splicing and promoter usage. Although homologous recombination studies have suggested that functional redundancy exists among the RARs, other studies have demonstrated that the various receptor subtypes may possess separate functions. Boylan and colleagues have recently reported that the inducibility of a number of retinoid target genes is differentially affected by the disruption of either the RARα or RARγ gene suggesting that these receptors are indeed not redundant, but each receptor regulates the expression of a set of unique genes.[14–16]

The RARs and RXRs have a motif consisting of six domains which are designated A to F (Figure 1a and b). The major



RARE/RXRE cis-Sequence Polymorphisms in selected genes

Type	Gene	Sequence
Direct Repeat (DR)-1	Mouse CRABP-II	GAAGGGCAGAGGTCACA
	Human ApoA1	GCAGGGCAGGGGTCAAG
	Mouse HHC1	TGAGGTCAGGGGTGGGG
	HBV	CGGGGTAAAGGTTCAGG
	Chicken Ovalbumin	TGGTGTCAAAGGTCAAA
	Rat ACO	CCAGGACAAAGGTCACG
	Rat PEPCK	CACGGCCAAAGGTCATG
Direct Repeat (DR)-2	Mouse CRBP-I	GTAGGTCAAAAGGTCAGA
	Mouse CRABP-II	CCAGTTCACCAGGTCAGG
	Mouse VL30-1	AAAGTTCATGTTTTCACA
	Mouse VL-30-2	TGGGGTGAAAAGTTTAGG
	Human ApoA1	AGGGGTCAAGGGTTCAGT
Direct Repeat (DR)-5	Human and mouse RARβ2	AGGGTTCACCGAAAGTTCACT
	Human RARα2	GAGGTTCAGCGAGAGTTCAGC
	Human RARγ2	CCGGGTCAGGAGGAGGTGAGC
	Mouse Hoxa-1	CAGGTTCACCGAAAGTTCAAG
	Mouse Hoxd-4	TAAGGTGAAATGCAGGTCACA
	Human MGP	AAGGTTCACCTTTTGTTCACC
	Human ADH3	AGGGGTCATTCAGAGTTCAGT
	Human Gα1	CAAGGGCAGGAGAAGGTCAGA

Figure 1 (a) RAR/RXR functional motifs and consensus sequences. (b) RARE/RXRE *cis*-sequence polymorphisms in selected genes.

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functional domains are the DNA binding domain, C, and the ligand-binding domain E. Important subdomains are those responsible for transactivation (AFs 1 and 2) and for dimerization (both located within the E domain). Activation of the RARs or RXRs requires the dimerization of these receptors.[17] While RARs may only dimerize with RXRs, RXRs can homodimerize as well as dimerize with the vitamin D, thyroid hormone and a number of orphan receptors.[17] Thus activation of RXRs can result in the signaling among numerous pathways. The dimeric receptors can then bind to target genes containing specific nucleotide sequences termed retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) which are located in the promoter regions of the DNA (Figure 1b). The RARs have been found to bind to a number of RAREs. In general, the RAREs have been found to be direct repeats of AGGTCA which are separated by five or two nucleotides; however they can be palindromic, inverted or more complex in nature.[7] RXR-RXR homodimers bind to RXREs (5′-AGGTCA-3′) in which the half sites are separated by a single base pair; more complex RXREs have also been identified.[18] The DNA/receptor dimer complex when bound to ligand results in a conformation change in the dimer complex resulting in either gene transcriptional activation or repression and subsequent modulation of mRNA levels. While the receptor binding downstream (usually the RAR) appears to play the dominant role in terms of the transcriptional response, the RXR is not a silent partner and can also modulate the transactivation process.[19–22]

RARs and RXRs can also modulate gene function indirectly through their interactions with other transcription factors and thus modify their activities. The activator protein-1 (AP-1) complex consists of a dimer composed of members of the Jun-Fos family of proteins which in turn bind to AP-1 consensus sequences located in the promoter of genes. The cyclic AMP response elements binding protein (CBP) binds to Jun in the AP-1 dimer to stimulate transcription from AP-1 sites. In the presence of retinoids, the retinoid receptors form a complex with CBP and thus inhibit its ability to activate the AP-1 complex.[23] CBP has also been found to function as a coactivator in RAR and RXR signaling.[24]

The isolation and crystallization of the various classes and subclasses of retinoids have allowed for the synthesis of both class and subtype-selective retinoids. RARα, β and γ as well as RXR selective retinoids have been reported by a number of groups.[19,25–31] These retinoids display selectivity in terms of their binding and transactivation of the retinoid receptor classes and subclasses. The advantages of these receptor selective retinoids, when compared to the natural retinoids, include their enhanced stability, reduced toxicity and activity in cells which display resistance to RA and 9-*cis*-RA.

Retinoids and leukemia

The ability of retinoids to influence the growth or induce differentiation of solid tumors growing *in vivo* is still controversial. However, the effect of these compounds on the growth and differentiation of acute promyelocytic leukemia (APL) cells has been demonstrated by numerous groups.[32–36] APL cells have been demonstrated to possess a t(15;17)(q22;q12–21) translocation resulting in the fusion of RARα receptor to the nuclear transcription factor (PML).[37,38] The RARα nuclear receptor has been felt to play a vital role in the differentiation of the APL cell to the neutrophil.[39–41] Recent experimental observations would suggest that RARα is not involved in enhancing myeloid differentiation, but rather functions as a repressor of the myeloid pathway and that this repression is relieved upon binding of RA to RARα.[42,43] This conclusion is supported by the finding that RARα-deficient mice possess normal numbers of terminated neutrophils in both the peripheral blood and bone marrow components.[44] There does, however, appear to be mild abnormalities in the myeloid pathway in these mice in terms of its response to a number of known stimulators of the myeloid pathway.[42] Overall the data would indicate that in the absence of ligand, RARα suppresses differentiation along the myeloid pathway and the recruitment of other precursor cells into this pathway. When ligand is present, RARα stimulates these processes. Generation of the PML-RARα fusion product results in the subsequent arrest of myeloid differentiation at the promyelocyte stage even in the presence of physiological concentrations of RA.[45] These promyelocytes are able to proliferate and thus lead to the subsequent development of leukemia. That the PML-RAR fusion gene plays a major role in the generation of APL as has been demonstrated in transgenic mice.[46] However, recent data would strongly suggest that the role of PML-RARα in the onset of APL is multifactorial and not simply through the inhibition of RARα function (Ref. 43 and Refs within). PML-RARα is capable of repressing both RA-dependent and independent genes in the myeloid precursor cells (Ref. 43 and Refs within). The etiology for the PML-RARα inhibition of normal RARα function remains unclear. Whether the RARα-mediated inhibition is a result of PML-RARα functioning as a dominant negative protein and thus blocking RARα activation of gene transcription, inhibiting the formation and function of novel macromolecular organelles termed PML oncogenic domains, reversing RAR inhibition of AP-1-mediated transcription or tightly binding histone deacetylases and thus inhibiting gene transcription is unclear.[47–53] Exposure of the APL cells to pharmacologic doses of RA results in the reversal of the PML-RAR inhibition of myeloid differentiation associated with enhanced RARα activation of promoters, inhibition of AP-1-mediated promoter activation and the formation of normal PML oncogenic domains.[51] Recent observations suggest that the enhanced binding of PML-RARα to the nuclear corepressor complex composed of N-CoR (nuclear receptor corepressor) or SMRT (silencing mediator for retinoid and thyroid-hormone receptors) with subsequent recruitment of Sin 3A and histone deacetylase HDAC1 or HDAC2 results in the inability of physiological concentrations of RA to disassociate PML-RARα from this complex. Several investigators have now demonstrated that the PML-RARα and PLZF-RARα fusion products have the unique ability to associate with multiple units of corepressor complexes, thus resulting in the transcriptional repression of a number of genes that are regulated in a RA-dependent and -independent fashion (Ref. 43 and Refs within, and Refs 54, 55). This results in the continued silencing of RARα-mediated and RARα-independent transcription and thus may play a major role in the inhibition of RA-mediated differentiation of these cells.[43,56] Several studies have provided further support for the hypothesis that PML-RARα repression of gene transcription through its interaction with HDACs plays a major role in PML-RARα inhibition of myeloid differentiation. Ferrara *et al*[57] demonstrated that addition of the HDAC inhibitor trichostatin A to RA results in the differentiation of AML cells (French–American–British classification M2, M4 and M5) which display resistance to differentiation by RA alone. Similarly, mice with APL and expressing PML-RARα or PLZF-RARα fusion proteins were utilized to demonstrate that treatment with HDAC inhibitors together with RA abrogated

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aberrant transcription mediated by the RARα oncoproteins and resulted in the induction of leukemia remission in mice where RA alone had minimal to no effect.[58] Interestingly, treatment of prostate carcinoma cells with the combination of 13-*cis*-RA and phenylbutyrate, a HDAC inhibitor, also resulted in the enhanced tumor cell apoptosis both *in vitro* and *in vivo* over that noted with each agent alone.[59] Thus the enhanced binding of the RARs to HDACs and transcription silencing complexes may occur in a number of malignant tissue types. The addition of histone deacetylase inhibitor along with retinoid may markedly enhance the therapeutic spectrum and activity of retinoids.

RA therapy of patients

Treatment of APL patients has been shown to induce complete remission and increase the survival of over 90% of the patients.[32,33] Complete remissions were obtained within 40 to 60 days.[32,33] While primary resistance to RA is rare, the duration of the complete remissions obtained with RA in these patients is brief with an average duration of 3 to 6 months.[32,34,60] Relapse is often associated with the acquisition of resistance to RA-mediated differentiation.[35,36,60,61] The etiology for this resistance to RA-mediated differentiation appears to be multifactorial. Marked reduction in the maximal achievable plasma concentration of RA have been noted approximately 1 to 2 weeks following the initiation of therapy. This appears to be partly due to RA-mediated enhanced cytochrome P-450 expression and activity and secondary RA catabolism by P-450 and lipoxygenase enzymes.[62–64] Other investigators have suggested RA-enhanced expression of cytosolic retinoic acid binding protein II (CRABP II) as a cause for the intrinsic APL cell resistance to RA.[63,65–67] Exposure to RA has been found to result in enhanced CRABP II mRNA and protein levels secondary to RA activation of nuclear RARs and their binding to and transactivation of the CRABP II promoter.[23,24,64] Overexpression of CRABP II would result in elevated levels of RA binding to CRABP II with subsequent sequestration of RA in the cytoplasm, enhanced RA metabolism by oxidative enzymes and thus decreased ligand transport to the PML-RARα nuclear receptor. Conflicting data have been presented regarding the role of CRABP II in acquired APL resistance to RA-mediated differentiation. Delva *et al*[68] found that high levels of CRABP II were found in four patients at relapse, but CRABP II protein could not be detected in patients' cells prior to RA therapy suggesting a role for elevated CRABP II in acquired RA resistance. However, a recent study by Zhou *et al*[64] found high expression of CRABP II in APL cells prior to RA therapy and more importantly, no correlation between acquired RA resistance and levels of CRABP II in the APL cells was noted. The discrepancy between this study and that of Delva *et al*[68] is difficult to explain. It should be added that while Delva *et al*[68] could discriminate between CRABP I and CRABP II, the assay utilized by Zhou *et al*[64] did not allow for this discrimination. Whether this is the explanation for the difference in results remains to be determined. It is clear that sustained exposure to RA results in the acquisition of resistance. Recent studies have suggested that pulse therapy with RA in APL patients may alleviate the acquired resistance observed in these patients, allowing for sustained remissions on RA therapy.[69] Decreased sensitivity of APL cells to RA-induced differentiation *in vitro* has been found in the cells isolated from patients following relapse on RA therapy;[36,44] missense mutations in the RARα region of the PML-RARα fusion gene were found in the APL cells in three out of 11 of these patients perhaps, resulting in the inability of RA to appropriately interact with PML-RARα and reverse its inhibitory effect on myeloid differentiation.[70] Thus RA binding to the RARα fusion protein and the subsequent degradation of this oncoprotein is an essential step in the RA-mediated differentiation process.

Novel retinoids

N-(4-hydroxyphenyl) retinamide (4-HPR): role of retinoid receptors

A number of novel retinoids have now been synthesized which induce cell death (apoptosis) utilizing a unique and as yet unidentified mechanism(s). 4-HPR (Figure 2) was one of 87 retinoids screened in a series of *in vitro* assays. 4-HPR was found to possess significant activity in reversing the keratination of vitamin A-deficient tracheal organ cultures.[25] Subsequent studies demonstrated that 4-HPR was an extremely active inducer of apoptosis, significantly less potent than RA in terms of the induction of differentiation and an important chemopreventive agent for a number of malignancies. Whether 4-HPR utilizes the retinoid nuclear receptor(s) in its induction of cell death remains unclear. Initial evidence suggesting that 4-HPR may exert its effect through a pathway not involving the RARs or RXRs was generated by several investigators. Delia *et al*[71] examined the effect of 4-HPR on a number of malignant hematopoietic cell lines, some of which were resistant to RA-mediated inhibition of growth. Growth of the cell lines HL-60 and Do HH2, both of which possess a functional RARα, was inhibited by RA, as well as 4-HPR. However, growth of the RA-resistant HL-60R cells which possess a 50 amino acid deletion in the E domain of RARα significantly inhibits the binding of RA, and the RA-resistant cell line NB306 which possesses decreased PML-RARα levels was inhibited only by 4-HPR, but not by RA.[71,72] The investigators concluded from these studies that 4-HPR and RA exert their cellular effects through different pathways and 4-HPR may induce growth inhibition and apoptosis independent of RAR expression.[71] Sheikh *et al*[73] arrived at similar conclusions when they examined the effect of 4-HPR on the growth of a number of breast carcinoma cell lines displaying sensitivity and resistance to RA-mediated growth inhibition. These investigators found that 4-HPR was more potent than RA as an anti-

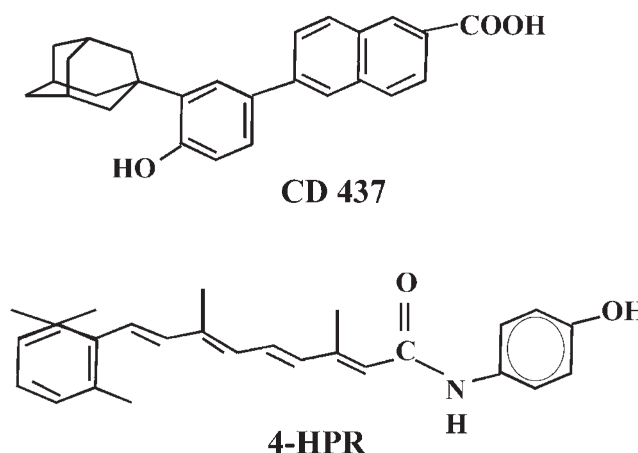

Figure 2 Structure of 4-HPR and CD437.

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proliferative agent and inhibited the growth of RA-resistant human breast carcinoma cells. Exposure to 4-HPR resulted in growth inhibition and DNA fragmentation with subsequent apoptosis in both RA sensitive and refractory cell lines.[73] N-(4-methoxyphenyl) retinamide (4-MPR), the major metabolite of 4-HPR, had no effect on cell growth. More importantly, these investigators found that 4-HPR and 4-MPR bound poorly to RARα, β and γ *in vitro* and only minimally activated RAREs and RXREs in human breast carcinoma cells.[73] Neither 4-HPR nor 4-MPR were metabolized to any of the known retinoids which are known to activate the RAREs or RXREs. In addition, while RA exhibited anti-AP-1 activity in the breast carcinoma cells, no anti-AP-1 activity was exhibited by 4-HPR.[73]

However, a potential role for the RARs in 4-HPR-mediated apoptosis has been supported by a number of other investigators. Fanjul *et al*[74] demonstrated that 4-HPR was a potent transactivator of RARγ and a moderate activator of RARβ utilizing transactivation assays of exogenously transfected RARs in CV-1 and MCF-7 cells. Concentrations of 5 to 20 μM 4-HPR were required to demonstrate significant transactivation of RARγ by 4-HPR. The question remains whether 4-HPR itself activated RARγ or a yet to be identified metabolite of 4-HPR was responsible for the observed transactivation of RARγ. These investigators also found that 4-HPR selective activation of RARγ appeared to be enhanced on some response elements (RAREs) but reduced on others when compared to that of natural retinoids. In addition, optimal RARγ activation was found at 4-HPR concentrations at which 4HPR induces apoptosis of cells.[74] Why these results differ from those reported by Sheikh *et al*[73] is unclear but cell type specific factors may play a role. Other studies have also suggested that the RARβ and RARγ retinoid receptors may play a role in 4-HPR-mediated cellular differentiation and apoptosis. Swisshelm *et al*[75] found that RARβ expression is enhanced in normal human mammary cells following exposure to 4-HPR. Several other reports have documented that RARβ or RARγ expression enhances 4-HPR-mediated events. Sabichi *et al*[76] reported that when RARβ expression was enhanced in an ovarian cell line following the stable transfection of a RARβ expression vector, 4-HPR-mediated inhibition of cellular proliferation was increased. In addition, among 12 human gynecologic cancer cell lines, there was a positive correlation between 4-HPR-mediated inhibition of cellular proliferation and RARβ expression. Similar results were reported by Liu *et al*[77] in examining the effect of 4-HPR on lung and gastric adenocarcinoma cell lines. These investigators found that 4-HPR induced growth inhibition correlated with RARβ2 expression, and loss of RARβ2 expression resulted in resistance to 4-HPR-mediated events.

Expression of RARγ has also been found to be associated with sensitivity to 4-HPR in a number of cell types. Sensitivity of the A2780 human ovarian carcinoma cell to 4-HPR-mediated apoptosis appeared to be increased, not only due to enhanced RARβ expression, but also due to elevated RARγ expression.[78] Clifford *et al*[79] made the interesting observation that while the apoptotic effect of 4-HPR on the F-9 embryonal carcinoma cell line was not inhibited by loss of RARγ, RXRα or both, the ability of 4-HPR to induce the differentiation of the F-9 cell line to a primitive endodermal differentiated phenotype was lost in the RARγ-Null cell line. Further support for a RAR involvement has been generated by a series of studies demonstrating inhibition of 4-HPR events by RAR antagonists.[80–82]

The results from all of these studies would suggest multiple pathways for 4-HPR mediated apoptosis, some involving RARβ or RARγ and other pathways functioning independently of these receptors. Certainly, activation of these RAR receptors could be indirect, perhaps through their phosphorylation. Although receptor activation may contribute to the overall aspects of 4-HPR-induced differentiation and apoptosis, the induction of apoptosis, however, appears to be predominately RAR independent in most of the cells examined.

4-HPR-mediated apoptosis

A number of other pathways have been implicated by which 4-HPR induces apoptosis. The ability of 4-HPR to generate radical oxygen species has been suggested to be an initial and important event in the induction of apoptosis by 4-HPR. The addition of 4-HPR to the human cervical carcinoma cell line C33A resulted in a 1.85-fold to 4.5-fold increase in reactive oxygen species approximately 2 h following exposure to 4-HPR.[83] The addition of radical oxygen scavengers inhibited the generation of the radical oxygen species, as well as 4-HPR induced apoptosis. Interestingly, 4-HPR was much less effective in generating reactive oxygen species and in inducing apoptosis in normal human cervical epithelial cells.[83] Sun *et al*[80] reported similar results following the exposure of the LNCaP prostate carcinoma cell line to 4-HPR. Delia *et al*[84] also found that the addition of antioxidants to the HL-60 human leukemia cell line suppressed the 4-HPR apoptotic effect and significantly prolonged survival. Utilizing flow cytometric and spectrofluorometric analysis, and the oxidation-sensitive probe 2′, 7′-dichloro fluorescin diacetate, the immediate and sustained production of intracellular free radicals, most likely hydroperoxides, were detected following exposure of HL-60 cells to 4-HPR; free radical survengers blocked the generation of hydroperoxides. Suzuki *et al*[85] found that thenoyltrifluoroacetone, a mitochondrial respiratory chain complex II inhibitor and carbonylcyanide-m-chlorophenyl hydrazone which uncouples electron transfer and ATP synthesis and inhibits radical oxygen species generation by the mitochondrial respiratory chain markedly inhibited 4-HPR-induced radical oxygen species generation. These results, as well as others, suggested that 4-HPR enhanced radical oxygen species generation by affecting a target between complex II and III, presumably coenzyme Q. This in turn results in the release of cytochrome C into the cytoplasm triggering caspase activation, DNA fragmentation and cell death.

The exposure of a large variety of cells to 4-HPR has resulted in a plethora of documented 4-HPR-mediated cellular events. Whether any of these are necessary for the induction of apoptosis in these cells remains unclear. More importantly, many of these events appear to be cell type-specific. The ability of 4-HPR to down-regulate c-Myc expression and telomerase activity has been documented by several investigators.[86,87] The ability of 4-HPR to down-regulate the expression of a number of important cell regulatory proteins including cyclin D1 and p34/cdc 2 has been found in a number of cell types but not in others which readily undergo apoptosis following exposure to 4-HPR.[88,89]

4-HPR: clinical relevance

It is important to discern the underlying mechanism(s) by which 4-HPR mediates its effects since 4-HPR has now been demonstrated to have significant chemopreventative activity against head and neck squamous carcinoma, ovarian adenocarcinoma and breast adenocarcinoma. Approximately 2800

women following surgery for localized breast carcinoma ($T_1N_oM_0$) were randomized to placebo *vs* 4-HPR treatment to assess whether 4-HPR prevented the development of second breast malignancy.[90] The trial displayed a possible beneficial effect to 4-HPR in premenopausal women but interestingly, an opposite trend in post-menopausal women.[90] The same trial also demonstrated that 4-HPR inhibited the development of ovarian carcinoma in premenopausal women.[91] The efficacy of 4-HPR to prevent relapse or the occurrence of oral carcinoma was examined in patients with oral leukoplakia following local resection; 115 patients were randomized following resection of the leukoplakia to 4-HPR or no intervention. During the first year of therapy, 12 local relapses or new lesions occurred in the control group and only three were observed in the 4-HPR group.[92] More importantly, patients tolerated 4-HPR extremely well with minimal side-effects. This is in contrast to the patients treated with 13-*cis*-RA who experienced a plethora of toxicities.

CD437: role of RARs

As previously discussed, a number of receptor-selective retinoids have been synthesized to further characterize the participation of receptor subclasses in retinoid-mediated events. One of these retinoids was 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN; CD437) which was originally synthesized as a RARγ-selective retinoid, but also displayed binding to RARβ, minimal binding to RARα and no binding to the RXRs (Figure 2).[27,93] Studies suggested that CD437, while binding to the RARs and thus a retinoid, may indeed exert some or all of its effects through pathways not involving the RARs.[94] This retinoid not only inhibited the growth of breast carcinoma cell lines which displayed sensitivity to RA-mediated inhibition of growth such as MCF-7 cells, but it also inhibited the growth and induced apoptosis in the human breast carcinoma cell lines (such as MDA-MB-231) which displayed resistance to these RA-mediated events.[94] While markedly inhibiting the growth of the MDA-MB-231 cells, CD437 did not activate the endogenous RAR or RXR nuclear receptors which were activated following incubation of the MDA-MB-231 cells with RA or an RXR selective retinoid (SR11246), respectively.[94] In addition, CD437 did not inhibit AP-1 activity.[94] Further evidence suggesting a RAR/RXR-independent pathway was provided by the observation that CD437 induced rapid apoptosis in the HL-60R which lack a functional RAR.[95] In general, the observations of other investigators have also supported the hypothesis that CD437 may utilize a RAR/RXR-independent pathway. Sun *et al*[96] found that CD437 induced apoptosis in a number of human non-small cell lung carcinoma cell lines which were resistant to RA-mediated growth inhibition and that RAR antagonist did not block the CD437 effect. Similar results and conclusions were obtained when these same investigators examined the response of normal, premalignant and malignant human bronchial epithelial cells to CD437.[97]

Several other studies have however suggested that RARs may be involved in CD437-mediated events. Sun *et al*[98] reported that CD437 may exert its effects on human head and neck squamous cell carcinoma cells by at least two distinct mechanisms: (1) an RAR-mediated suppression of squamous differentiation which was found to be inhibited by RAR antagonist; and (2) an RAR-independent induction of apoptosis. Holmes *et al*[99] made the interesting observation that the RAR antagonist MM11253 reduced CD437-mediated apoptosis of CA-OV3 and SK-OV3 ovarian carcinoma cells and that the overexpression of RARγ enhanced the CD437 effect. That the potential role of the RARs in CD437-mediated apoptosis may be cell type-specific is supported by our observation that CD437-mediated apoptosis of HL-60R cells does not require gene transcription or protein synthesis.[100] We found that CD437-mediated poly (ADP-ribose) polymerase (PARP) cleavage and apoptosis of HL-60R cells occurred in the presence of, or absence of, either actinomycin D or cycloheximide.[100] Thus activation of RARs and subsequent gene transcription appears to be not required.

CD437-mediated cell cycle arrest and apoptosis

One of the initial events following exposure to CD437 is cell cycle arrest.[94] We initially reported the rapid onset of G1 cell cycle arrest following exposure of human breast carcinoma cells to CD437.[94] The onset of G1 cell cycle arrest was preceded by the enhanced expression of the cyclin/cyclin dependent kinase complex inhibitor $p21^{WAF1/CIP1}$.[94] The induction of $p21^{WAF1/CIP1}$ expression by CD437 is through a p53-independent mechanism and has been found to occur at the post-transcriptional level involving a unique element located in the 3′-untranslated region (3′-UTR) of the messenger RNA.[101] Exposure to CD437 results in the stabilization of the $p21^{WAF1/CIP1}$ message with enhanced expression of $p21^{WAF1/CIP1}$ protein, inhibition of a number of cyclin/cyclin-dependent kinase activity and subsequent G1 arrest.[94,101] In a number of other cell types, while increased $p21^{WAF1/CIP1}$ levels are noted, the cells do not arrest in the G1 phase of the cell cycle, but in the S phase of the cell cycle.[102,103] In normal mammary cell lines, this arrest in S phase is associated with inhibition of the cyclin A/cdk2 cyclin-dependent kinase.[103] Cyclin A/cdk phosphorylates the E2F-1/DP-1 transcription factor complex resulting in its inability to bind to the E2F-1 consensus sequence. Failure to phosphorylate the E2F-1/DP-1 complex in early S phase and thus inhibit E2F-1 binding to its consensus sequence in this crucial period of the cell cycle, has been shown to result in S phase cell cycle arrest and subsequent apoptosis.[104–106]

Elucidation of the crucial initial steps by which CD437 induces apoptosis remains to be discerned. Modulation of a number of important mediators of the apoptotic process has been suggested by certain groups as playing an important role. The important tumor suppressor gene, p53, has been implicated to play a role in CD437-mediated apoptosis in certain cell types. Sun *et al*[107] reported that CD437 up-regulated the expression of the DR5 death receptor in three human non-small cell lung carcinoma cell lines with wild-type p53, but not in five cell lines possessing a mutant p53. Degradation of p53 in the H460 cell line by transfection with the human papilloma virus 16 EG (HPV-16) gene, blocked CD437-mediated enhancement of p53 and DR5 expression and, more importantly, inhibited CD437-mediated G1 arrest and apoptosis in these cells. The importance of the DR4 and DR5 death receptors in CD437-mediated apoptosis in human lung cancer cells was emphasized by the observation that the addition of the TRAIL, the ligand for DR-4 and DR-5 receptors, enhanced CD437-mediated apoptosis in those human lung carcinoma cell lines possessing a wild-type p53.[108] These investigators also made the observation that exposure of the human lung carcinoma cells possessing a wild-type p53 to CD437 resulted in the up-regulation of both Fas mRNA and protein levels and that the addition of Fas ligand also syner-

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gized with CD437 in the induction of apoptosis in these cells.[109] The roles of p53, Fas and DR4 and DR5 in CD437-mediated apoptosis appear to be cell type-specific, since they could not be found in a number of other cell types.[110]

Activation of the mitogen-activated protein kinase (MAPK) kinase pathway has also been found as an early event following the exposure of a number of cell types to CD437. Activation of both the p38 and JNK protein kinases is noted within 2 h following the addition of CD437 to HL-60 cells.[100] Activation of p38 appears to require caspase activation in these cells while activation of JNK appears not to require caspase activation.[100] Inhibition of p38 kinase activity does not inhibit CD437-mediated apoptosis; whether JNK kinase activation is required for the induction of apoptosis by CD437 remains to be discerned. Evidence supporting a role for JNK activation in CD437-mediated apoptosis was presented by Li *et al*[111] who found that expression of a dominant negative c Jun inhibited CD437-mediated apoptosis in lung carcinoma cells.

As has been noted with a number of other agents, the induction of apoptosis by CD437 involves the inhibition of mitochondrial function and the disruption of the mitochondrial transmembrane potential in a variety of cells. Recently, Marchetti *et al*[112] have suggested that this is a direct effect of CD437 on the mitochondria. These investigators found that exposure of cytoplasts (anucleate cells) of the RPMI 8226 multiple myeloma cell line to CD437 results in the rapid disruption of the mitochondrial transmembrane potential, release of cytochrome c and generation of radical oxygen species. More importantly, exposure of purified mitochondria to CD437 also results in disruption of the transmembrane potential; this is not seen following the exposure of the purified mitochondria membrane to RA. Utilizing a cell-free *in vitro* system consisting of isolated nuclei and mitochondria supernatants and treatment with CD437 or placebo, only the supernatants from the mitochondria treated with CD437 underwent chromatin condensation; this result would suggest a direct interaction between CD437 and mitochondria as the initial event in the CD437-mediated apoptosis. The observations of Marchetti *et al*[112] are extremely provocative and exciting, but several issues regarding their applicability to other malignant cells can be raised. The results of Marchetti *et al*[112] would suggest that cells exposed to CD437 should quickly undergo apoptosis. Similarly, Zhang *et al*[113] recently reported that CD437-mediated apoptosis in the HL-60 cell lines involves a direct interaction between CD437 and lysosomes with the rapid release of cathepsin D from lysosomal vesicles with the subsequent generation of free radicals, activation of caspases and the apoptotic pathway. However, there appears to be a wide spectrum in terms of the duration of CD437 exposure required for the induction of apoptosis. HL-60 and HL-60R cells undergo apoptosis within hours of exposure to CD437,[114] while other leukemic cell lines and breast and prostate carcinoma cell lines require up to 4 days of exposure to undergo apoptosis. Thus, the simple exposure of CD437 to the mitochondria or to lysosomes does not provide an explanation for this wide variation in time required for CD437-mediated apoptosis. How CD437 induces apoptosis in a number of malignant cell types still remains to be discerned. Recently, the ability of CD437 to bind to a nuclear protein(s) with different ligand specificity than the RARs or RXRs was detected.[115] Neither RA, 9-*cis*-RA or 13-*cis*-RA was able to compete with CD437 for binding to this protein. Binding to this protein was characterized by two components; a high affinity phase $K_D = 2.8$ nM, $\beta_{max} = 89$ fmols/mg protein and a low affinity phase = $K_D = 1.5\ \mu$M, $\beta_{max} = 29\,000$ fmol/mg protein.[115] Whether this protein has any role in CD437-mediated apoptosis is currently under investigation.

CD437 and its analogs when given to tumor-bearing animals has resulted in a significant inhibition of tumor growth. Nude mice bearing palpable melanoma tumors were treated with CD437 using oral gavage.[116] Treatment with this retinoid resulted in a marked reduction (greater than 50%) in tumor growth. Analysis of the tumors following treatment with CD437 revealed a marked increase in c-Fos mRNA expression and the induction of apoptosis as indicated by TUNEL staining.[116] Whether this therapy was associated with any toxicity to the animals was not described. CD437 treatment of an ovarian carcinoma xenograft (PEO4) grown in nude mice resulted in a significant ($P < 0.05$) growth inhibition.[117] While a dose of 30 mg per kg was toxic, the mice appeared to tolerate a dose of 20 mg per kg. Even more dramatic effects were observed by the Pfahl group utilizing a number of CD437 analogs.[118,119] These investigators found that the treatment of lung and breast xenograft models with a number of CD437 analogs resulted in greater than 80% reduction in the tumor volumes when compared to the non-treated mice. In addition, the mice appeared to display minimal to no toxicity from the therapy. Ponzanelli *et al*[120] recently reported that CD437 is highly active in a APL-derived NB4 SCID mouse model with a five-fold decrease in the leukemic blasts in the CD437 treated mice and a 2.5-fold increase in survival time.

Conclusion

The role of RA in the treatment of APL has been well-delineated. The fact that the therapeutic efficacy of RA has been restricted to the treatment of APL may now be modified by the observation that the addition of HDAC inhibitors in the the presence of RA has demonstrated significant activity both *in vitro* and *in vivo* against a number of different acute myelogenous leukemia FAB subtypes. Clinical trials utilizing these combination of agents offer the possibility of exciting clinical results, as well as expanding our knowledge in the area of the potential interactions between the RARs and the HDACs.

The development and the investigations of the novel retinoids 4-HPR and CD437 has been exciting, both in the studies examining the underlying pathways by which these agents induce apoptosis in malignant cells and their potential therapeutic efficacies in the treatment of a number of malignant diseases. Delineating the underlying mechanism(s) by which these compounds exert their activity although challenging, will allow for the identification of new mediators of cell growth and apoptosis. Novel analogs of CD437 which do not transactivate the RARs or RXRs have now ben synthesized and their modulation of cellular pathways involved in growth suppression and apoptosis are being actively investigated.[121] These analogs should provide new insights into the unique mechanisms utilized by these compounds.

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SPOTLIGHT

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