Heterocycle-Containing Retinoids. Discovery of a Novel Isoxazole Arotinoid Possessing Potent Apoptotic Activity in Multidrug and Drug-Induced Apoptosis-Resistant Cells

Daniele Simoni,*,† Marinella Roberti,‡ Francesco Paolo Invidiata,§ Riccardo Rondanin,† Riccardo Baruchello,† Cinzia Malagutti,† Angelica Mazzali,† Marcello Rossi,† Stefania Grimaudo,# Francesca Capone,# Luisa Dusonchet,⊥ Maria Meli,⊥ Maria Valeria Raimondi,# Marilena Landino,⊥ Natale D'Alessandro,⊥ Manlio Tolomeo,# Dhar Arindam,⊗ Shennan Lu,⊗ and Doris M. Benbrook⊗

Dipartimento di Scienze Farmaceutiche, Via Fossato di Mortara 17−19, Università di Ferrara, 44100 Ferrara, Italy; Dipartimento di Scienze Farmaceutiche, Università di Bologna, Bologna, Italy; Dipartimento Farmacochimico Tossicologico e Biologico, Università di Palermo, Palermo, Italy; Divisione di Ematologia e Servizio AIDS, Policlinico di Palermo, Palermo, Italy; Dipartimento di Scienze Farmacologiche, Università di Palermo, Palermo, Italy; and Department of Obstetrics and Gynecology and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, P.O. Box 26901 WP2470, Oklahoma City, Oklahoma 73190

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In a search for retinoic acid (RA) receptor ligands endowed with potent apoptotic activity, a series of novel arotinoids were prepared. Because the stereochemistry of the C9-alkenyl portion of natural 9-*cis*-RA and the olefinic moiety of the previously synthesized isoxazole retinoid **4** seems to have particular importance for their apoptotic activity, novel retinoid analogues with a restricted or, vice versa, a larger flexibility in this region were designed and prepared. The new compounds were evaluated in vitro for their ability to activate natural retinoid receptors and for their differentiation-inducing activity. Cytotoxic and apoptotic activities were, in addition, evaluated. In general, these analogues showed low cytotoxicity, with the restricted structures being slightly more active than the more flexible ones. As an exception, however, the isoxazole retinoid **15b** proved to be particularly able to induce apoptosis at concentrations $<5\ \mu$M, showing a higher activity than the classical retinoids such as *all-trans*-RA, 13-*cis*-RA, and 9-*cis*-RA and the previously described synthetic retinoid **4**. **15b** also exhibited a good affinity for the retinoid receptors. Interestingly, another important property of **15b** was its ability to induce apoptosis in the HL60R multidrug-resistant (MDR) cell line, at the same concentration as is effective in HL60. Therefore, **15b** represents a new retinoid possessing high apoptotic activity in an MDR cell line. The ability of **15b** to act on K562 and HL60R cells suggests that this compound may have important implications in the treatment of different leukemias, and its structure could offer an interesting model for the design of new compounds endowed with apoptotic activity on MDR- and retinoid-resistant malignancies.

Introduction

Retinoids are a class of natural and synthetic vitamin A analogues structurally related to *all-trans*-retinoic acid (ATRA, **1**) (Figure 1).[1] They are involved in the physiology of vision and as morphogenic agents during embryonic development.[1] They are known to play a major role in regulating the growth and differentiation of a wide variety of normal and malignant cell types and can inhibit cell proliferation and induce differentiation and apoptosis at the cellular level.[2a−e] The induction of apoptosis is related to cell growth and differentiation in various ways, depending on the cell type.[3a,b] Retinoids exert most of their effects by binding to specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each of which is encoded by three separate genes designated α, *β*, and *γ*.[1,4] These receptors form RXR−RAR heterodimers that regulate transcription by binding to RA response elements (RAREs) in the promoters of retinoid-responsive genes.[1] ATRA binds and activates the RARs, whereas 9-*cis*-retinoic acid (9-*cis*-RA) (**2**) binds and activates both RARs and RXRs.[1] Owing to their ability to regulate aberrant cell growth, retinoids are currently being evaluated as preventive or therapeutic agents in a variety of human premalignancies and cancer.[5a−g] Encouraging preliminary clinical results have also demonstrated the importance of retinoids in combination chemotherapy of cancer.[5a,6] Indeed, retinoids may increase the activity of other biologic or chemotherapeutic agents, thus offering new opportunities for the development of effective combination regimens. Moreover, by inducing apoptosis, they may overcome tumor resistance to conventional anticancer agents. In current oncologic practice, ATRA is being used to induce remission in acute promyelocytic leukemia (APL) patients.[7]

The ability of retinoids to regulate cellular processes in vivo is unfortunately associated with a high incidence of undesiderable side effects. These include toxicity in

* Corresponding author (telephone +39-0532-291291; fax +39-0532-291296; e-mail smd@dns.unife.it).
† Università di Ferrara.
‡ Università di Bologna.
§ Dipartimento Farmacochimico Tossicologico e Biologico, Università di Palermo.
# Policlinico di Palermo.
⊥ Dipartimento di Scienze Farmacologiche, Università di Palermo.
⊗ University of Oklahoma Health Sciences Center.

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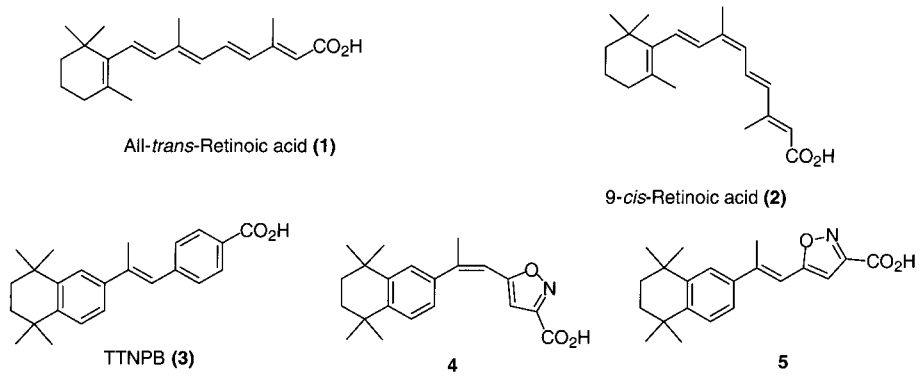


Figure 1. Natural and synthetic retinoids.

skin and mucous membranes, hyperlipidemia, skeletal side effects, visual disturbances, and teratogenicity.[2b,8−12] Thus, one way to expand the therapeutic use of retinoids is not only to increase the efficacy but also to reduce the toxicity of the compounds.[13,14] In this regard, as stated before, retinoids interact with a diverse range of receptor subtypes to produce their wide spectrum of effects. Therefore, it is reasonable to think that it may be possible to design selective retinoid receptor subtype agonists devoid of dangerous side effects. Moreover, apoptosis is a major modality by which tumor cells can be eliminated, so the identification of new retinoids able to induce apoptosis in different tumor cell types, independent or not of their differentiating activity, can be an important goal in cancer therapy and may provide new useful tools for the treatment of patients with retinoid-resistant APL or other malignancies.

The premises for our new work on retinoids were formulated by preliminary studies carried out in our laboratory. Indeed, we recently demonstrated that some new isoxazole-containing retinoids are endowed with interesting differentiating properties against human APL HL60 cells.[15,16] We have also evaluated many synthetic heterocyclic isoxazole retinoids by cell growth inhibition and differentiation assays and also with respect to their effects on cell cycle progression.[17] We found that a novel G1 phase-targeting compound **4** (Figure 1), which is structurally related to arotinoids, was endowed with apoptotic activity. This derivative bearing the cis configuration at the double bond may resemble 9-*cis*-RA but, unlike this natural compound, does not bind the retinoid receptors. We found that **4** was able to induce apoptosis in HL60 cells after only 24 h of treatment. The percentages of apoptotic cells in cultures treated with compound **4** were 23, 60, and 90% after 24, 48, and 72 h, respectively. This apoptosis-inducing activity was 6.5 and 4 times higher than those of 13-*cis*-RA and 9-*cis*-RA, respectively, whereas ATRA at the concentration of 50 μM was unable to induce apoptosis. Interestingly, the trans isomer **5** was only a poor inducer of apoptosis but retained an appreciable differentiating activity in HL60 cells. Thus, we have considered **4** as a possible new lead for the development of novel apoptosis-inducing agents structurally related to retinoids and targeted to the cell cycle. Here, we describe our results on the synthesis and biological activity of some new isoxazole-containing arotinoids structurally related to the aromatic retinoid (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-propenyl]benzoic acid (TTNPB or Ro13-7410, **3**).[1,5d] All of the new retinoids were tested for their differentiating, cytotoxic, and apoptotic activities. In addition, the abilities of the compounds to regulate the retinoid receptors in vitro were evaluated using transcriptional activation assays.

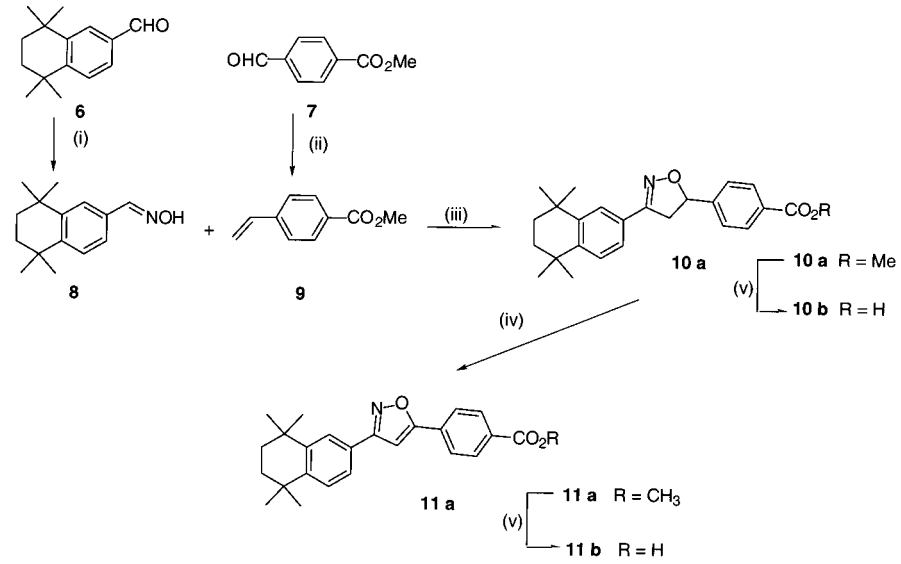
This study describes, for the first time, the novel heterocyclic isoxazole retinoid **15b** (Scheme 1) that contains an isoxazole heterocycle replacing the alkenyl portion of TTNPB and is able to induce apoptosis in HL60 cells after only 24 h of treatment at a concentration of 10 μM. The compound **15b** is also a potent inducer of apoptosis in leukemia cell lines resistant to drugs and to retinoic acid, such as the multidrug-resistant variant of HL60, HL60R, the *BCR-ABL* oncogene-expressing K562 leukemia, and the HUT78 T-cell lymphoma. Therefore, these findings further support our hypothesis that heterocyclic analogues of arotinoids such as **15b** can possess apoptotic activity and may provide an interesting new class of drugs for the therapy of cancer, especially leukemias resistant to conventional treatments.

Design and Chemistry

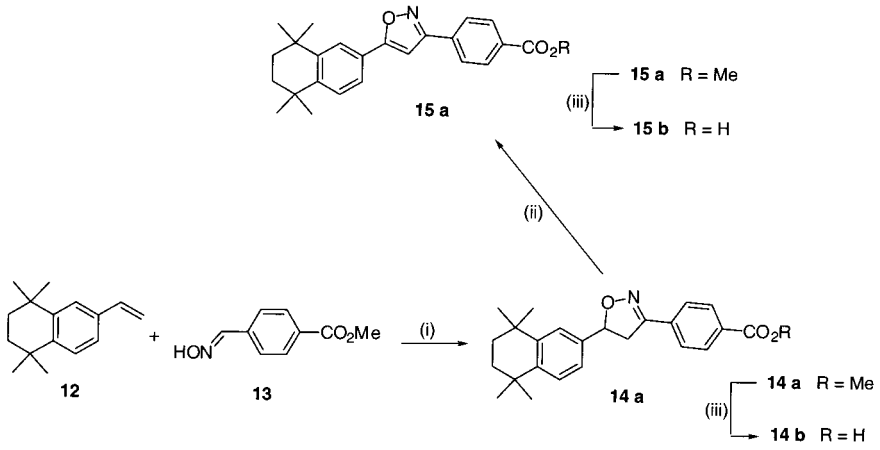
As our lead **4** may be considered to be a heterocyclic analogue of TTNPB (**3**), we utilized the structure of this known arotinoid as the basis for the design of novel retinoids endowed with apoptotic activity.[18] Because the stereochemistry of the C9-alkenyl portion of 9-*cis*-RA, which corresponds to the only olefinic moiety in **4**, seemed to be of particular importance for apoptotic activity, we planned the synthesis of new retinoid analogues having varied flexibility in this region. Thus, the alkenyl motif of TTNPB was replaced by an isoxazole (compounds **11b** and **15b**) or an isoxazoline ring (compounds **10b** and **14b**) (Scheme 1), to restrict the molecule, or by a flexible amino- or oxymethyl group, to enable the system to better "fit" the receptor as in analogues **17e**−**h** and **20b** (Scheme 2). Although compounds **17e** and **20b** were known from patent literature,[19] their biological activities were not described.

Because inclusion of a heteroatom in the arotinoid ring reduces the toxicity 1000-fold and inclusion of a heteroatom in the ring of *trans*-retinoic acid reduces the toxicity 3-fold,[13,14,20] we considered that introduction of a heterocycle in place of the alkenyl portion of TTNPB, such as in the isoxazole and the isoxazoline systems, might similarly produce compounds endowed with retinoid-like activity but with concomitant reduced toxicity. In this regard it is worth noting that heteroaroti-

Scheme 1



(i): NH_2OH x HCl, $NaHCO_3$; (ii) $CH_3P(Ph)_3{}^+Br^-$, NaH; (iii) $CHCl_3$, Pyridine, *N*-Chlorosuccinimide,TEA; (iv): CCl_4, NBS; (v): LiOH, H_2O.

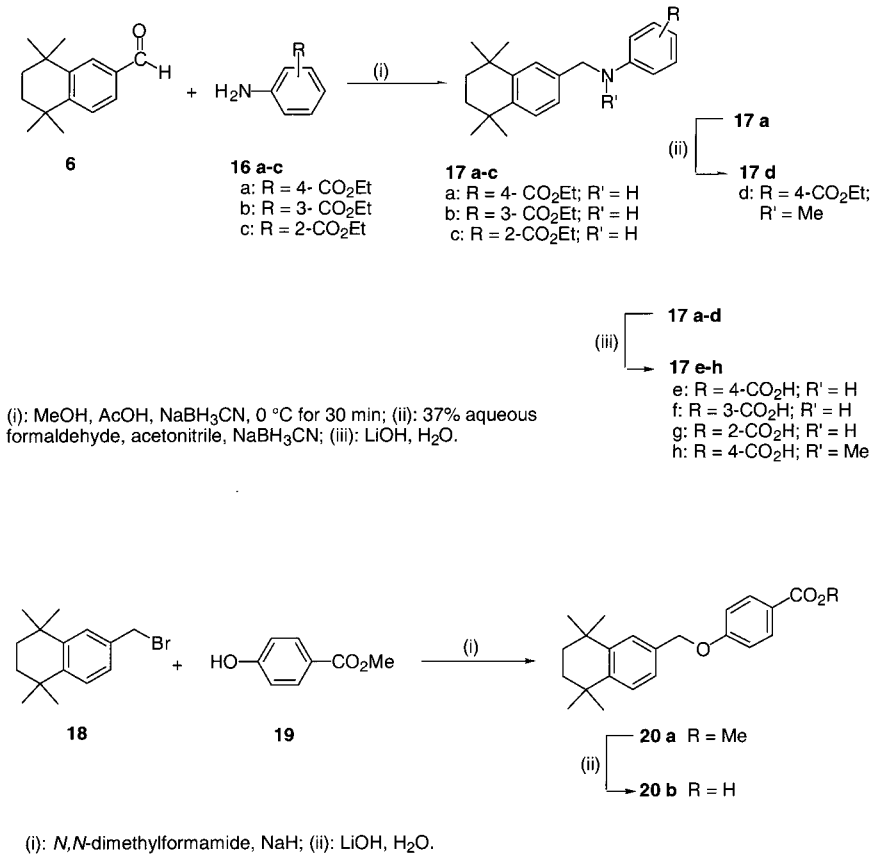


(i): $CHCl_3$, Pyridine, *N*-Chlorosuccinimide,TEA; (ii): CCl_4, NBS; (iii): LiOH, H_2O.

noids, a class of TTNPB analogues containing at least one heteroatom in the partially saturated ring, were less toxic than arotinoids and have demonstrated promise as anticancer agents.[14] The reason for their reduced toxicity appears to be due to a single structural alteration, namely, inclusion of a heteroatom in the bicyclic ring. Our heterocyclic retinoids **10b**, **14b**, **11b**, and **15b**, however, retain some structural features of **4**, that is, the heterocycle and tetrahydrotetramethylnaphthalenyl rings. On the other hand, analogues **17e**−**h** and **20b** contain an acyclic heteroatom and the tetrahydrotetramethylnaphthalenyl ring. We speculated that such new retinoid analogues might retain the apoptotic activity of **4**.

The syntheses of the isoxazoline and isoxazole derivatives **10b**, **14b**, **11b**, and **15b** are outlined in Scheme 1. We took advantage of known procedures for the procurement of aldehyde **6**,[21] whereas aldehyde **7** was commercially available. Reaction of **6** and **7** with hydroxylamine in an MeOH/H_2O (3:1) solution produced the corresponding oximes **8** and **13** in high yields. Wittig reaction between carbomethoxybenzaldehyde **7** and methyltriphenylphosphonium bromide, in the presence of NaH, readily produced olefin **9** in 80% yield. Olefin **12** was also easily obtained through a similar Wittig reaction on aldehyde **6**. The nitrile oxide generated from oxime **8** following Torssell's procedure[22] underwent a [3 + 2] regioselective cycloaddition with alkene **9** to produce isoxazoline **10a** in good yield. In the same manner, the isoxazoline **14a** was obtained in lower yield from the nitrile oxide generated from oxime **13** and alkene **12**. Oxidation of the isoxazoline rings of **10a** and **14a** was easily accomplished by reaction with *N*-bromosuccinimide to produce the corresponding 3-bromo derivatives, which were then dehydrobrominated by triethylamine in methylene chloride to the desired isoxazoles **11a** and **15a** in 85% overall yield. Upon treatment with lithium hydroxide in aqueous methanol, isoxazolines **10a** and **14a**, as well as the isoxazoles **11a** and **15a**, underwent facile hydrolysis of the ester functions to give, after treatment with aqueous HCl, the acids **10b**, **14b**, **11b**, and **15b**.

Scheme 2



Preparation of flexible retinoids **17e**−**h** and **20b** used standard procedures (Scheme 2). Acids **17e** and **20b** were prepared as reported, and some of the steps for **17a**−**c** and **20a** followed known procedures.[19] The synthesis of amines **17a**−**c** involved reductive amination using sodium cyanoborohydride of the imine produced by reaction of aminobenzoates **16a**−**c** and aldehyde **6**. The *N*-methyl derivative **17d** was prepared by reaction of secondary amine **17a** with formaldehyde and sodium cyanoborohydride. Ether **20a** was prepared by alkylation of the phenoxide of 4-hydroxybenzoate **19** by benzylic bromide.

Biological Results and Discussion

The object of this work was to investigate the importance of the C9 double bond in conferring apoptotic activity to synthetic retinoids. The main aim was to develop retinoids endowed with potent anticancer activity. In this context, it is worth noting that interesting results were recently described regarding structural modifications at the C9 double bond in 9-*cis*-RA as well as in TTNPB.[23−25] We have prepared a novel class of C9−C10 conformationally restricted TTNPB analogues **10b**, **11b**, **14b**, and **15b** (Scheme 1) bearing an isoxazole or isoxazoline ring in place of the alkenyl portion of this molecule. Moreover, **17e**−**h** and **20b** (Scheme 2) with a more flexible moiety were also prepared. These retinoids were assayed in vitro for cell growth inhibition and the ability to induce apoptosis in HL60 cells.

Our experiments showed that the treatment of HL60 cells for 48 h with **10b**, **11b**, **14b**, or **17e**−**h** had only low inhibitory effects, with IC_{50} values in the range of 40−80 μM, whereas oxymethylene **20b** was devoid of activity (Table 1). Analogues **17e**−**h** were able to induce apoptosis at 40−60 μM. Interestingly, growth inhibitory and apoptotic effects of **15b** on HL60 cells were markedly greater than those of the other analogues (Figures 2a,b and 3 and Table 1). It was able to induce apoptosis at concentrations <10 μM (Figure 2b) and thus was also more active than the classical retinoids ATRA, 13-*cis*-RA, and 9-*cis*-RA and isoxazole **4** (Figure 4).

Table 1. Cell Growth Inhibition Activity Expressed as IC_{50} and Apoptotic Activity Expressed as AC_{50} (Concentration Able To Induce 50% Apoptosis) of Compounds **10b**, **11b**, **14b**, **15b**, **20b**, and **17e**−**h** in HL60 Cells[a]

compound	IC_{50} (μM)	AC_{50} (μM)
10b	68 ± 15	>100
11b	80 ± 21	>100
14b	75 ± 8	>100
15b	1 ± 0.4	5 ± 2.6
20b	>100	>100
17h	58 ± 9.6	95 ± 13
17e	40 ± 2.9	70 ± 7.2
17g	49 ± 8.7	88 ± 6.7
17f	60 ± 6.9	99 ± 15
ATRA	18 ± 1.2	>100

[a]Evaluation after 48 h of treatment.

At a concentration of 1 μM, **15b** was unable to induce apoptosis after 24−48 h. However, after 6−7 days of exposure, almost all of the cells showed morphological aspects of apoptosis (data not shown). Thus, it is worth noting that, by transposing aryl groups at the 3,5-positions of the isoxazole ring from those of **11b** to **15b**, we may greatly enhance activity. Another important property of **15b** was its activity against drug-resistant cells and cells resistant to classical retinoids (ATRA, 13-*cis*-RA, and 9-*cis*-RA). For example, at concentrations

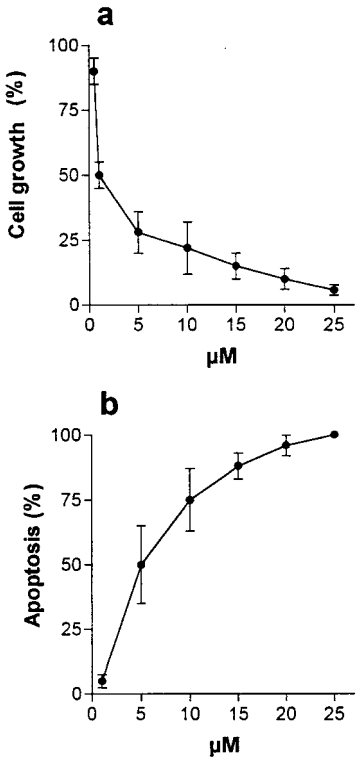


Figure 2. Cytotoxic (a) and apoptotic (b) activities of **15b** in HL60 leukemia cells. Cytotoxicity was evaluated by the trypan blue dye exclusion test and apoptosis by a morphological analysis (see Material and Methods). Data are the mean ± SD of five independent experiments.

effective against HL60, **15b** was able to induce apoptosis in HL60R, an MDR cell line derived from HL60 that overexpresses the multidrug efflux pump P-glycoprotein (Table 2). In the same manner, **15b** induced apoptosis in K562, an apoptotic agent-resistant leukemia cell line expressing the *BCR-ABL* oncogene, and in T-cell lymphoma HUT78 (Table 2).

Compounds **10b**, **11b**, **14b**, and **17e** were also evaluated in vitro for their ability to activate natural retinoic acid receptors and for their differentiation-inducing activity. The ability of the compounds to activate endogenous retinoid receptors was evaluated in a vulvar carcinoma cell line, SW962, which expresses all of the known human RARs and RXRs. Figure 5 demonstrates the receptor expression profile of this cell line in comparison to other squamous carcinoma cell lines. The SW962 cell line expresses RARα, RARγ, RXRα, and RXRβ at levels similar to those of the other cell lines. RARβ, however, which is often lost or decreased during tumorigenesis, is expressed at a low level in the SW962 cell line. The SW962 cell line was stably transfected with the reporter plasmid, RARE-*tk*-CAT, which contains an RARE linked to a thymidine kinase (*tk*) promoter that drives a chloramphenicol acetyl transferase (CAT) gene. This RARE is found in the RARβ gene promoter. The retinoid receptors bind to the RARE and thereby regulate expression of the CAT gene. Retinoids that activate a receptor will cause an increase in CAT protein expression in a concentration-dependent manner. These compounds induced a concentration-dependent increase in CAT expression, demonstrating that the endogenous receptors in the SW962 cell line are activated to transactivate the RARE (Table 3).

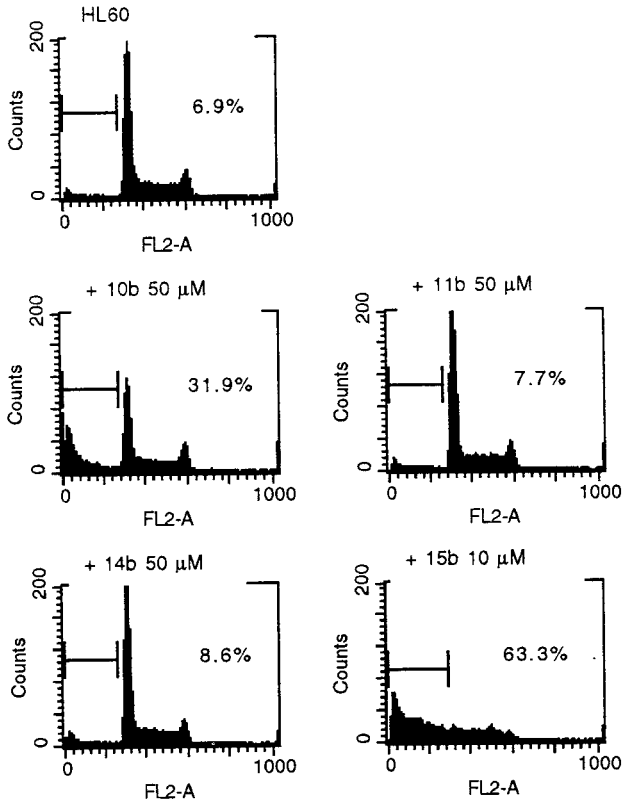


Figure 3. Flow cytometry analysis of apoptosis in HL60 cells treated for 48 h with **10b**, **11b**, **14b**, or **15b**. The percentage of apoptotic events accumulating under the marker in the pre-G_0G_1 phase of the cell cycle was calculated using CellQuest software (Becton Dickinson).

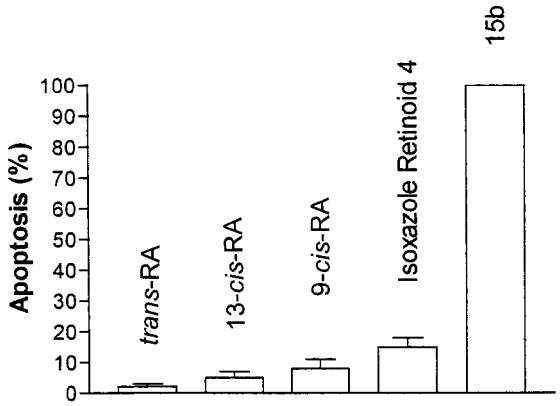


Figure 4. Percent apoptosis induced by *trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, and isoxazole **4** or **15b** at the concentration of 30 μM in HL60 leukemia cells. Evaluation was performed after 48 h of treatment. Data are the mean ± SD of five independent experiments.

Transactivation efficacy was determined by dividing the fold RARE induction in cultures treated with 10 μM synthetic retinoids by the fold induction in cultures treated with 10 μM 9-*cis*-RA and multiplied by 100. Compound **15b** induced the highest activity, which was 97% of that induced by 9-*cis*-RA. To achieve this high level, however, micromolar concentrations were required. Their potency was derived from dose−response curves by determining the concentration of each compound that induced half-maximal activation (EC_{50}). The more potent compounds, therefore, exhibit lower EC_{50} values. The high EC_{50} value of **15b** is due to the sudden increase in activity between 1 and 10 μM (Figure 6). At

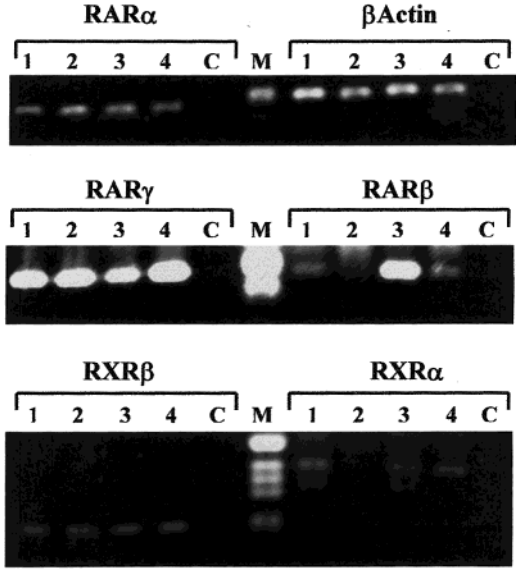


Figure 5. Expression of retinoid receptors in tumor cell lines. Reverse transcriptase Polymerase Chain Reaction (RT-PCR) was performed on RNA isolated from two vulvar carcinoma cell lines, SW962 (lane 1) and A431 (lane 2), and two head and neck carcinoma cell lines, SCC-2 (lane 3) and SCC-38 (lane 4). Primers that specifically amplify the RARα, RARβ, RARγ, RXRα, and RXRβ retinoid receptor genes or the β actin gene as a control for RNA quantity and quality were used as indicated above the lanes. The reaction products were electrophoresed on 1.5% agarose gels containing ethidium bromide as pictured above. Reactions performed in the absence of RNA were also electrophoresed (lane C) as negative controls. Lane M contains DNA molecular weight markers, with the highest band on the RAR gels on the left being 201 base pairs and the highest band in the RXR gels on the right being 505 base pairs followed by 396, 344, 288, 220, 201, 154, 134, and 75. The middle picture was overexposed to show the weak RARβ bands. The sizes for the amplified bands are ~200 base pairs except for RXRα, which is 400 base pairs.

Table 2. Cell Growth Inhibition Activity Expressed as IC_{50} and Apoptotic Activity Expressed as AC_{50} of **15b** in Retinoic Acid-Resistant Cell Lines HL60R, K562, and HUT78[a]

cell line	IC_{50} (μM)	AC_{50} (μM)
HL60R	1.4 ± 0.5	4.2 ± 2.2
K562	2.3 ± 1.2	4.8 ± 3.4
HUT78	0.8 ± 0.2	1.5 ± 0.8

[a] Evaluation after 48 h of treatment; these cell lines were resistant to ATRA (IC_{50} and AC_{50} > 100).

Table 3. Efficacy and Potency of βRARE Transactivation by **10b**, **11b**, **14b**, **15b**, **17e**, and 9-*cis*-RA in SW962 Cells

compound	efficacy[a] (%)	potency[b] (nM)
10b	46.5	2860
11b	48.0	129
14b	26.3	961
15b	97.6	10000
17e	64.5	671
9-*cis*-RA	100.0	16.9

[a] The efficacy was derived by dividing the fold RARE induction at 10 μM retinoid by the fold induction at 10 μM 9-*cis*-RA (8-fold induction) and multiplying by 100. [b] The potency or concentration required to induce half of the maximal activity (EC_{50}) was derived from the dose−response curves in Figure 6.

lower concentrations, **15b** is equally as effective as the other compounds.

We also evaluated the differentiation-inducing effects of **10b**, **11b**, **14b**, and **15b** in different leukemia cell lines. Their differentiating activity in HL60 was, however, lower than that shown by ATRA or 9-*cis*-RA (Table 4). The compounds were completely inactive as differentiating agents in RA-resistant K562 and HL60R leukemia and in the HUT78 lymphoma cell lines (data not shown).

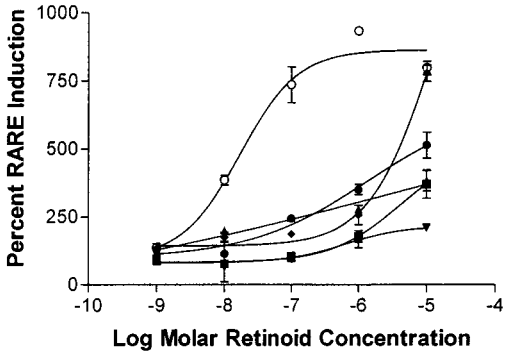


Figure 6. Activation of endogenous retinoic acid receptors in a vulvar carcinoma cell line SW962: (■) **10b**; (▲) **11b**; (▼) **14b**; (◆) **15b**; (●) **17e**; (○) 9-*cis*-RA. Cultures of cell line SW962 containing the RARE-*tk*-CAT reporter plasmid were treated with each concentration of compound for 24 h, and the amount of CAT expression in lysates of treated cells was quantitated. The fold RARE induction was determined by dividing the amount of CAT protein in the treated cultures by the amount in the control cultures.

Table 4. Differentiating Activity of Compounds **10b**, **11b**, **14b**, and **15b** in HL60 Cells[a]

compound	CD14[b] (%)	CD11b[c] (%)	morphology
10b	42.8	0	monocytes
11b	38.6	0	monocytes
14b	32.5	0	monocytes
15b			(apoptosis)
ATRA	0	98.2	granulocytes

[a] Evaluation after 6 days of exposure to each retinoid at a concentration of 5×10^{-5} M. [b] Monocyte-associated antigen. [c] Granulocyte-associated antigen.

Our results indicate that introduction of a flexible moiety in place of the alkenyl portion of TTNPB produces compounds of low interest as apoptosis inducers in leukemia cells. A difference exists between the aminomethyl and the oxymethyl moieties, in that the ether **20b** is devoid of growth inhibitory activity, whereas the amines retain this activity. In contrast, we found that one isoxazole analogue (**15b**) had high retinoid transactivation activity accompanied by apoptotic activity. Because natural and synthetic retinoids contain an sp^2 hybridized side chain, we may argue that the inactivity of the isoxazolines may be due to the partially hydrogenated heterocyclic ring, and therefore it is likely that more structural resemblance to natural retinoids is needed for transactivation to occur. Moreover, the location of the substituents on the isoxazole in **11b** and **15b** seems to be the basic premise in conferring activity. Thus, **15b** appears to be a useful tool to understand the physiological role of retinoid receptors in induction of apoptosis.

It will be important to determine if **15b** exhibits selective activation of RAR or RXR subtypes and to identify the mechanisms through which it induces apoptosis. This study demonstrates that **15b** can regulate gene transcription through natural RA receptors. Dawson et al. reported the biological activity and receptor selectivity of 4,5-dihydroisoxazole **10b**, which activated RARs with EC_{50} values in the nanomolar or

less range.[26] The structural similarity of **10b** to the RXR-selective methyloxime of 4-(4,5,6,7-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)benzoic acid may be the reason for RXR activation.[27] The significantly lower EC_{50} values reported by Dawson et al. in comparison to this study may be explained by the methods used to evaluate receptor activation. Whereas Dawson et al. used a reporter construct that contained two copies of the TREpal DNA binding site, this study used the βRARE DNA binding site. The type of DNA binding site used in receptor transactivation studies has been shown to strongly effect the results obtained.[28] Evaluating transcriptional regulation by using endogenous receptors in reporter cell lines, as in this study, differs significantly from the receptor co-transfection assays performed to determine receptor selectivity. The former method has the benefit of being more biologically relevant, because the receptors studied are at natural levels. The disadvantage of this method is that the receptor selectivity cannot be determined without using cell lines in which endogenous receptor subtype protein levels vary. The disadvantages of the co-transfection method are its greater expense and artifacts induced by the unnaturally high receptor levels. The higher levels of individual receptor expression in the co-transfection assays result in EC_{50} values lower than those observed for endogenously expressed receptors in reporter cell line assays. For instance, in our studies, the EC_{50} value of 9-*cis*-RA is 16.9 nM, which is ~17-fold higher than reported in the co-transfection assay.[26] Furthermore, compounds with increased receptor selectivity may exhibit EC_{50} values in reporter cell line assays higher than strong pan-agonists such as 9-*cis*-RA. Therefore, it is expected that receptor co-transfection studies of the compounds in this paper will have lower EC_{50} values for individual receptors than those observed for an endogenous receptor mixture in the reporter cell line assay. Studies of the receptor specificity of these compounds are proposed in our plans to understand the molecular mechanism and to develop **15b** for clinical applications.

The roles of individual RA receptors observed in apoptosis have varied depending on the retinoids and cell lines studied.[29,30] In support of this theory, Fenretinide (4-HPR) can induce differentiation in F9 mouse teratocarcinoma cells in a receptor-dependent manner at 1 μM,[31] whereas at 3−12.5 μM, this compound induces apoptosis in a variety of cell lines.[31−37] The finding that 4-HPR can induce apoptosis in RAR-null cells indicates that its molecular mechanism of apoptosis induction is independent of RA receptor activation.[31] Other promising novel retinoids, such as CD437, and some heteroarotinoids also induce apoptosis through receptor-independent mechanisms in addition to their receptor-dependent activities.[38−40] The ability of the compounds in this paper to induce apoptosis, but not differentiation, in retinoid-resistant cell lines also suggests that their mechanism of apoptosis may be receptor independent, whereas their mechanism of differentiation is receptor dependent.

K562 is a cell line expressing the Philadelphia chromosome, which is the product of a reciprocal exchange between the long arms of chromosome 9 and 22, resulting in a hybrid gene, in which the amino-terminal sequence of the *bcr* gene on chromosome 22 is fused to the second exon of the *c-abl* gene on chromosome 9. The resulting *BCR-ABL* protein is an oncoprotein that confers resistance to apoptosis induced by etoposide, actinomycin D, cycloheximide, and dexamethasone.[41] The ability of **15b** to act in this cell line as in *BCR-ABL*-negative cell lines implies that it may be an effective treatment for chronic myelogenous leukemia and acute lymphoblastic (ALL), as well as nonlymphoblastic (ANLL) leukemias expressing the *BCR-ABL* oncogene. Also, the ability of **15b** to induce apoptosis in HL60R cells represents an important indication for its possible clinical use. We have previously observed that HL60R is resistant to drug-induced apoptosis independently of its expression of P-glycoprotein. This resistance seems to be correlated to the presence of high constitutive levels of activated transcription factor NF-kB, which is not observed in the sensitive parental HL60.[42]

In summary, isoxazole arotinoid **15b** represents a novel retinoid endowed with apoptotic activity in MDR cells. Its ability to act in K562 and HL60R cell lines suggests that it may have important implications in the treatment of different leukemias. Thus, **15b** may be a drug useful in both MDR- and apoptosis-resistant malignancies.

Experimental Section

Chemistry. General Methods and Materials. Melting points were obtained in open capillary tubes and are uncorrected. Reactions and product mixtures were routinely monitored by thin-layer chromatography (TLC) on Merck silica gel precoated F_{254} plates. Infrared spectra (IR, cm^{-1}) were measured on a Perkin-Elmer 257 instrument. Nuclear magnetic resonance (^1H NMR, δ) spectra were determined, when not specified, in $CDCl_3$ solution using a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as the internal standard; *J* values are expressed in hertz. Light petroleum ether refers to the 40−60 °C boiling range fractions. Column chromatographies were performed with Merck 60−200 mesh silica gel. All drying operations were performed over anhydrous magnesium sulfate. Column chromatography (medium pressure) was carried out by using the "flash" technique. Microanalysis of all new synthesized compounds agreed within ±0.4% of calculated values.

General Procedure for Oximes 8 and 13. To a solution of hydroxylamine hydrochloride (250 mg, 3.7 mmol) dissolved in water (7 mL) was added $NaHCO_3$ (470 mg, 5.6 mmol) portionwise at 0 °C, and the mixture was stirred for 30 min at room temperature. The appropriate aldehyde **6** or **7** (3.1 mmol), dissolved in methanol (5 mL), was then added to the solution, and stirring was continued for an additional 6 h. Methanol was evaporated in vacuo and the residue extracted with diethyl ether. The organic extracts were washed with brine, dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel (eluent, diethyl ether/light petroleum).

5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carbaldehyde oxime (8): yield 67%; mp 123−126 °C (diethyl ether/light petroleum); ^1H NMR 1.29 (s, 6H), 1.30 (s, 6H), 1.70 (s, 4H), 7.34 (s, 3H), 7.50 (s, 1H), 8.11 (s, 1H). Anal. ($C_{15}H_{21}NO$): C, H, N.

4-(Hydroxyiminomethyl)benzoic acid methyl ester (13): yield 80%; mp 119−122 °C (diethyl ether/light petroleum); IR (KBr) 1726, 1609, 1438, 1284, 1110, 966, 763; ^1H NMR 3.9 (s, 3H), 7.27 (s, 1H), 7.69 (d, 2H, J = 8.4), 8.06 (d, 2H, J = 8.3), 8.17 (s, 1H). Anal. ($C_9H_9NO_3$): C, H, N.

General Procedure for Olefins 9 and 12. NaH (80%) (145 mg, 6 mmol), previously washed with dry hexane, was added

to a stirred suspension of methyltriphenylphosphonium bromide (1.07 g, 3 mmol) in dry tetrahydrofuran (15 mL) containing the appropriate aldehyde **6** or **7** (3 mmol). After 5 h of stirring at room temperature, diethyl ether (30 mL) was added, and the mixture was poured into ice−water and extracted with Et_2O. The combined organic extracts were dried and evaporated, and the residue was chromatographed on silica gel (eluent, diethyl ether/light petroleum).

4-Vinylbenzoic acid methyl ester (9): yield 60%; oil; IR (neat) 1724, 1608, 1436, 1278, 1107, 782; ^{1}H NMR 3.91 (s, 3H), 5.38 (d, 1H, J = 10), 5.96 (d, 1H, J = 17.6), 6.75 (dd, 1H, J = 17.6, J = 10), 7.46 (d, 2H, J = 8), 7.99 (d, 2H, J = 8). Anal. ($C_{10}H_{10}O_2$): C, H.

1,2,3,4-Tetrahydro-1,1,4,4-tetramethyl-6-vinylnaphthalene (12): yield 67%; oil; ^{1}H NMR 1.27 (s, 6H), 1.29 (s, 6H), 1.68 (s, 4H), 5.17 (d, 1H, J = 10.8), 5.69 (d, 1H, J = 17.6), 6.68 (dd, 1H, J = 17.6, J = 10.8), 7.23−7.31 (m, 3H). Anal. ($C_{16}H_{22}$): C, H.

General Procedure for Isoxazolines 10a and 14a. A mixture of *N*-chlorosuccinimide (174 mg, 1.3 mmol), pyridine (2 drops), and oxime **8** or **13** (1.3 mmol) in anhydrous $CHCl_3$ (15 mL) was stirred for 1 h at 50−60 °C. Olefin **9** or **12** (1.4 mmol) was then added followed by triethylamine (0.27 mL, 1.95 mmol) in $CHCl_3$ (5 mL). After 20 min of stirring at 25 °C, water was added, and the organic phase was washed with 2.5% HCl and water, then dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel (eluent, ethyl acetate/light petroleum).

4-[3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-4,5-dihydroisoxazol-5-yl]benzoic acid methyl ester (10a): yield 63%; mp 138−140 °C (ethyl acetate/light petroleum); ^{1}H NMR 1.22 (s, 6H), 1.24 (s, 6H), 1.63 (s, 4H), 3.24 (dd, 1H, J = 16.6, J = 7.6), 3.77 (dd, 1H, J = 16.6, J = 11.1), 3.85 (s, 3H), 5.71 (dd, 1H, J = 11, J = 7.8), 7.20−7.42 (m, 4H), 7.60 (d, 1H, J = 1.5), 7.98 (dd, 2H, J = 6.7, J = 1.7). Anal. ($C_{25}H_{29}NO_3$): C, H, N.

4-[5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-4,5-dihydroisoxazol-3-yl]benzoic acid methyl ester (14a): yield 75%; mp 171−173 °C (ethyl acetate/light petroleum); IR (KBr) 1718, 1587, 1435, 1283, 1112, 916, 772; ^{1}H NMR 1.27 (s, 6H), 1.29 (s, 6H), 1.66 (s, 4H), 3.38 (dd, 1H, J = 16.5, J = 8.9), 3.76 (dd, 1H, J = 16.5, J = 11), 3.94 (s, 3H), 5.68−5.78 (m, 1H), 7.15 (d, 1H, J = 8.3), 7.31−7.35 (m, 2H), 7.77 (d, 2H, J = 8.4), 8.08 (d, 2H, J = 8.4). Anal. ($C_{25}H_{29}NO_3$) C, H, N.

General Procedure for Isoxazoles 11a and 15a. To a solution of the appropriate isoxazoline **10a** or **14a** (0.25 mmol) in carbon tetrachloride (10 mL) was added NBS (66 mg, 0.37 mmol), and the mixture was gently refluxed for 3 h. Hydrogen bromide was slowly liberated. The cooled solution was filtered from the precipitated succinimide and washed with 5% aqueous sodium hydroxide and then with water until the organic phase became clear. The organic layer was dried and the solvent removed in vacuo. The residue was chromatographed on silica gel (eluent, ethyl acetate/light petroleum).

4-[3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)isoxazol-5-yl]benzoic acid methyl ester (11a): yield 80%; mp 137−140 °C (ethyl acetate/light petroleum); IR (KBr) 1716, 1594, 1424, 1280, 1106, 771; ^{1}H NMR 1.33 (s, 6H), 1.36 (s, 6H), 1.74 (s, 4H), 3.97 (s, 3H), 6.94 (s, 1H), 7.42 (d, 1H, J = 8.1), 7.62 (dd, 1H, J = 8.1, J = 1.2), 7.83 (d, 1H, J = 1.2), 7.93 (d, 2H, J = 8.3), 8.16 (d, 2H, J = 8.3). Anal. ($C_{25}H_{27}NO_3$): C, H, N.

4-[5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)isoxazol-3-yl]benzoic acid methyl ester (15a): yield 60%; mp 140−143 °C (ethyl acetate/light petroleum); ^{1}H NMR 1.32 (s, 6H), 1.36 (s, 6H), 1.73 (s, 4H), 3.96 (s, 3H), 6.82 (s, 1H), 7.43 (d, 1H, J = 8.3), 7.58 (dd, 1H, J = 8.3, J = 1.7), 7.80 (d, 1H, J = 1.7), 7.96 (d, 2H, J = 8.4), 8.15 (d, 2H, J = 8.4). Anal. ($C_{25}H_{27}NO_3$): C, H, N.

General Procedure for Amines 17a—c. Sodium cyanoborohydride (0.31 g, 5 mmol) was added at 0 °C to a solution of the aldehyde **6** (1.08 g, 5 mmol) and the appropriate amine **16a**−**c** (5 mmol) in MeOH (20 mL) containing AcOH (0.2 mL). The mixture was stirred at room temperature for 30 min and then concentrated. The residue was treated with aqueous 5% $NaHCO_3$ (10 mL) and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried, and evaporated under reduced pressure. The residue was purified by chromatography (eluent, diethyl ether/light petroleum).

4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalen-2-ylmethylamino)benzoic acid ethyl ester (17a): yield 60%; mp 58−60 °C (diethyl ether/light petroleum); IR (KBr) 1691, 1611, 1520, 1455, 1286, 1178, 1107, 843, 769; ^{1}H NMR 1.30 (s, 12H), 1.37 (t, 3H, J = 7), 1.70 (s, 4H), 3.5 (br, 1H), 4.29−4.35 (m, 4H), 6.62 (d, 2H, J = 8.8), 7.12 (dd, 1H, J = 8.4, J = 1.4), 7.28−7.33 (m, 2H), 7.89 (d, 2H, J = 8.7). Anal. ($C_{24}H_{31}NO_2$): C, H, N.

3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalen-2-ylmethylamino)benzoic acid ethyl ester (17b): yield 80%; mp 45−46 °C (diethyl ether/light petroleum); ^{1}H NMR 1.29 (s, 12H), 1.39 (t, 3H, J = 7), 1.70 (s, 4H), 3.50 (br, 1H), 4.29 (s, 2H), 4.36 (q, 2H, J = 7), 6.81−6.88 (m, 1H), 7.16−7.39 (m, 6H). Anal. ($C_{24}H_{31}NO_2$): C, H, N.

2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalen-2-ylmethylamino)benzoic acid ethyl ester (17c): yield 67%; mp 40−42 °C (diethyl ether/light petroleum); ^{1}H NMR 1.27 (s, 12H), 1.38 (t, 3H, J = 7.1), 1.70 (s, 4H), 3.25 (br, 1H), 4.26−4.37 (m, 4H), 6.06−6.73 (m, 2H), 7.10−7.16 (m, 1H), 7.28−7.34 (m, 3H), 7.94 (dd, 1H, J = 8.0, J = 1.5). Anal. ($C_{24}H_{31}NO_2$): C, H, N.

4-(*N*-Methyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethylamino)benzoic acid ethyl ester (17d). Sodium cyanoborohydride (50 mg, 0.8 mmol) was added to a stirred solution of **17a** (183 mg, 0.5 mmol) in acetonitrile (5 mL), containing 37% aqueous formaldehyde (0.22 mL, 2.7 mmol). After 15 min of stirring at room temperature, the reaction was treated dropwise with glacial AcOH until the solution tested neutral. Stirring was continued for an additional 45 min, with occasional addition of glacial AcOH to maintain the pH near neutrality. The solvent was evaporated at reduced pressure, and 2 N KOH (10 mL) was added to the residue. The resulting mixture was extracted with diethyl ether. The combined ether extracts were washed with 0.5 N KOH, dried, and evaporated in vacuo. The residue was chromatographed on silica gel (eluent, diethyl ether/light petroleum): yield 80%; oil; ^{1}H NMR 1.22 (s, 6H), 1.26 (s, 6H), 1.36 (t, 3H, J = 7), 1.67 (s, 4H), 3.10 (s, 3H), 4.32 (q, 2H, J = 7), 4,57 (s, 2H), 6.71 (d, 2H, J = 9), 6.92 (dd, 1H, J = 8.2), 7.10 (s, 1H), 7.24 (d, 1H, J = 8.3), 7.9 (d, 2H, J = 9). Anal. ($C_{25}H_{33}NO_2$): C, H, N.

4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethoxy)benzoic acid methyl ester (20a). To a stirred solution of **19** (0.17 g, 1.1 mmol) in anhydrous *N*,*N*-dimethylformamide (10 mL) was added sodium hydride (0.1 g, 3.85 mmol) in a single portion at room temperature. The mixture was stirred for 10 min at room temperature, and then benzyl bromide **18** (0.62 g, 2.2 mmol) was added. Stirring was continued for another 2 h, diethyl ether (15 mL) was added, and the mixture was poured into ice−water. The extract was washed with brine, dried, and concentrated. The crude product was purified by silica gel column chromatography (eluent, diethyl ether/light petroleum): yield 56%; mp 152−154 °C (methanol); IR (KBr) 1717, 1608, 1510, 1263, 1166, 1106, 1043, 847, 766; ^{1}H NMR 1.25 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 3.89 (s, 3H), 5.04 (s, 2H), 7.01 (d, 2H, J = 8.9), 7.22 (dd, 1H, J = 8, J = 2.5), 7.33−7.35 (m, 2H), 8.00 (d, 2H, J = 8.8). Anal. ($C_{23}H_{28}O_3$): C, H.

General Procedure for Carboxylic Acids 10b, 11b, 14b, 15b, 17e—h, and 20b. A mixture of ester **10a**, **11a**, **14a**, **15a**, **17a**−**d**, or **20a** (1 mmol), methanol (10 mL), water (6−7 mL), and lithium hydroxide (40 mg, 1.5 mmol) was allowed to stand at 50−60 °C for 24 h. The solution was concentrated in vacuo to remove methanol, and the remaining aqueous solution was extracted with diethyl ether to separate trace amounts of unreacted ester. The aqueous solution was acidified with 1 M hydrochloric acid and extracted with three portions of ethyl acetate. [In the case of esters **17a**−**d**, the aqueous solution

was neutralized (pH 7) with 1 M hydrochloric acid and extracted with dichloromethane.] The combined organic extracts were washed with saturated aqueous sodium chloride and dried. Removal of the solvent under reduced pressure afforded a residue, which was chromatographed on silica gel (eluent, ethyl acetate/light petroleum).

4-[3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-4,5-dihydroisoxazol-5-yl]benzoic acid (10b): yield 84%; mp 129−132 °C (ethyl acetate); IR (KBr) 1686, 1612, 1458, 1420, 1285, 896; ^{1}H NMR 1.30 (s, 6H), 1.32 (s, 6H), 1.71 (s, 4H), 3.33 (dd, 1H, J = 16.6, J = 7.6), 3.87 (dd, 1H, J = 16.5, J = 11.1), 5.81 (dd, 1H, J = 11, J = 7.6), 7.34−7.54 (m, 4H), 7.68 (d, 1H, J = 1.1), 8.14 (d, 2H, J = 8.2), 11.44 (br, 1H). Anal. ($C_{24}H_{27}NO_3$): C, H, N.

4-[5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-4,5-dihydroisoxazol-3-yl]benzoic acid (14b): yield 88%; mp 219−221 °C (methanol); ^{1}H NMR 1.28 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 3.39 (dd, 1H, J = 16.7, J = 8.9), 3.77 (dd, 1H, J = 16.7, J = 11), 5.70−5.80 (m, 1H), 7.15 (dd, 1H, J = 8.2, J = 1.3), 7.31−7.35 (m, 2H), 7.80 (d, 2H, J = 8.4), 8.15 (d, 2H, J = 8.4), 12.15 (br, 1H). Anal. ($C_{24}H_{27}NO_3$): C, H, N.

4-[3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)isoxazol-5-yl]benzoic acid (11b): yield 80%; mp 239−241 °C (methanol); IR (KBr) 1689, 1598, 1424, 1292, 772; ^{1}H NMR (acetone-d_6) 1.33 (s, 6H), 1.37 (s, 6H), 1.76 (s, 4H), 5.63 (s, 1H), 7.51 (d, 1H, J = 8.2), 7.75 (dd, 1H, J = 8.2, J = 1.9), 7.95(d, 1H, J = 1.9), 8.08 (d, 2H, J = 8.4), 8.21 (d, 2H, J = 8.4), 11.21 (br, 1H). Anal. ($C_{24}H_{25}NO_3$): C, H, N.

4-[5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)isoxazol-3-yl]benzoic acid (15b): yield 74%; mp 218−220 °C (methanol); ^{1}H NMR 1.33 (s, 6H), 1.36 (s, 6H), 1.74 (s, 4H), 6.85 (s, 1H), 7.44 (d, 1H, J = 8.4), 7.60 (dd, 1H, J = 8.4, J = 1.2), 7.81 (d, 1H, J = 1.2), 8.02 (d, 2H, J = 8.2), 8.25 (d, 2H, J = 8.2), 11.58 (br, 1H). Anal. ($C_{24}H_{25}NO_3$): C, H, N.

4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethoxy)benzoic acid (20b): yield 80%; mp 199−201 °C (methanol); IR (KBr) 1681, 1605, 1512, 1427, 1253, 1167, 1043, 772; ^{1}H NMR 1.30 (s, 12H), 1.70 (s, 4H), 5.07 (s, 2H), 7.04 (d, 2H, J = 8.8), 7.21−7.24 (m, 1H), 7.34−7.36 (m, 2H), 8.08 (d, 2H, J = 8.8), 11.97 (br, 1H). Anal. ($C_{22}H_{26}O_3$): C, H.

4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethylamino)benzoic acid (17e): yield 90%; mp 207−209 °C (water); IR (KBr) 1661, 1603, 1530, 1457, 1280, 1181, 838, 774; ^{1}H NMR 1.28 (s, 6H), 1.29 (s, 6H), 1.70 (s, 4H), 3.52 (br, 1H), 4,33 (s, 2H), 6.63 (d, 2H, J = 8.3), 7.11−7.16 (m, 1H), 7.28−7.36 (m, 2H), 7.95 (d, 2H, J = 8.6), 12.55 (br, 1H). Anal. ($C_{22}H_{27}NO_2$): C, H, N.

3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethylamino)benzoic acid (17f): yield 80%; mp 151−153 °C (methanol); ^{1}H NMR 1.28 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 3.15 (br, 1H), 4,30 (s, 2H), 6.89 (dd, 1H, J = 7.5, J = 2.5), 7.15 (dd, 1H, J = 7.5, J = 2.5), 7.29−7.33 (m, 3H), 7.40−7.41 (m, 1H), 7.45 (d, 1H, J = 7.5), 12.45 (br, 1H). Anal. ($C_{22}H_{27}NO_2$): C, H, N.

2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethylamino)benzoic acid (17g): yield 70%; mp 197−199 °C (water); ^{1}H NMR 1.28 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 3.77 (br, 1H), 4.42 (s, 2H), 6.60−6.70 (m, 2H), 7.12 (dd, 1H, J = 7.3, J = 2.2), 7.30−7.31 (m, 1H), 7.37 (dd, 1H, J = 7.3, J = 2), 7.98 (dd, 2H, J = 7.5, J = 1.9), 12.62 (br, 1H). Anal. ($C_{22}H_{27}NO_2$): C, H, N.

4-(*N*-Methyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ylmethylamino)benzoic acid (17h): yield 80%; mp 261−263 °C (water); ^{1}H NMR (dimethyl sulfoxide-d_6) 1.16 (s, 6H), 1.19 (s, 6H), 1.60 (s, 4H), 3.07 (s, 3H), 4.59 (s, 2H), 6.74 (d, 2H, J = 8.9), 6.87 (dd, 1H, J = 8.1, J = 1.1), 7.12 (s, 1H), 7.23 (d, 1H, J = 8.1), 7.72 (d, 2H, J = 8.8), 12.10 (br, 1H). Anal. ($C_{23}H_{29}NO_2$): C, H, N.

Biology. Cell Cultures. HL60 and K562 cell lines were obtained from ATCC (Rockville, MD). HUT78 cells were obtained as gifts from Dr. Giovina Ruberti (CNR, Rome, Italy). HL60R were selected for multidrug resistance (MDR) by exposure to gradually increasing concentrations of daunorubicin and express P-glycoprotein. HL60 cells were grown in RPMI 1640 (Gibco, Grand Island, NY) containing 10% FCS (Gibco), 100 units/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), and 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO) in a 5% CO_2 atmosphere at 37 °C. SW962 cells were obtained from ATCC and were grown in Cellgro L15 media (Fisher Scientific, Pittsburgh, PA) containing 10% FBS (Sigma Chemical Co.) and antibiotic/antimycotic (Sigma Chemical Co.) in a 5% CO_2 atmosphere at 37 °C**.** Only lots of FBS containing undetectable (<8 nM) vitamin A, as determined by HPLC, were used.

Cell Growth Inhibition Assays. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25-mm wells (Costar, Cambridge, MA) in 1 mL of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was counted on a hemocytometer after staining with trypan blue to exclude dead cells and expressed as percent of control cell proliferation. IC_{50} (drug concentration able to induce a 50% cell growth inhibition) and AC_{50} (drug concentration able to induce apoptosis in 50% of cells) were calculated after exposure of cells to increasing concentrations of each drug for 48 h.

Analysis of Cellular Differentiation. Cells (2×10^5) were exposed to each retinoid and after 6 days were examined for induction of differentiation by morphology and flow cytometry. The morphology of the cells was evaluated from cytospin slide preparations stained with May Grunwald-Giemsa stain solutions. The expression of monocytic (CD14) or granulocytic (CD11c) cell surface antigens was studied by a two-color direct immunofluorescence staining technique. Cells were stained using FITC-conjugated mouse anti-human CD14 and PE-conjugated anti-human CD11c mouse monoclonal antibodies (both from ORTHO Diagnostic System, Raritan, NJ). Control studies were performed with negative murine isotype antibodies. Analysis of fluorescence was performed using a FACSort instrument (Becton Dickinson, Mountain View, CA).

Morphological Evaluation of Apoptosis and Necrosis. Apoptosis and necrosis were determined morphologically by fluorescent microscopy after labeling with acridine orange and ethidium bromide. Cells (2×10^5) were centrifuged (300*g*), and the pellet was resuspended in 25 μL of the dye mixture. The suspensions of 10 μL were placed on a microscope slide and covered with a 22-mm^2 coverslip and examined in oil immersion with a 100× objective using a fluorescent microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labeling of the cells with ethidium bromide.

Flow Cytometry. Cells were washed twice with ice-cold PBS. To determine apoptosis and cell-cycle phase distribution, the cells were resuspended at 1×10^6/mL of a hypotonic fluorochrome solution containing propidium iodide [50 mg/mL in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40]. After 1 h of incubation, the samples were filtered through 40-μm nylon mesh cloth, and fluorescence was analyzed as single-parameter frequency histograms using a FACSort instrument. Apoptosis was determined by evaluating the percentage of events accumulating in the pre-G_0G_1 position.

Receptor Activation. Cultures of the SW962 cell line that had been stably transfected with the RARE-*tk*-CAT reporter gene were treated with each compound at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M in dimethyl sulfoxide (DMSO). A control culture was treated with the same volume of DMSO that was used in the treated cultures. After 24 h of treatment, cellular extracts were prepared and assayed for CAT protein using an ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). The amount of CAT in each extract was normalized by dividing by the protein concentration in each extract that was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Each treatment was performed in triplicate, and the normalized CAT values were averaged and used to determine the fold RARE induction. The fold RARE induction was derived by

dividing the amount of CAT protein in the treated cultures by the amount in the control culture. The efficacy was derived by dividing the activity (fold RARE induction) of the highest concentration of each compound by the activity of the highest concentration of 9-*cis*-RA (8-fold induction) and multiplying by 100. The potency or concentration required to induce half of the maximal activity (EC_{50}) was derived from dose−response curves using GraphPad Software. The experiment was repeated twice with similar results.

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