Structure−Activity Relationship Studies of Novel Heteroretinoids: Induction of Apoptosis in the HL-60 Cell Line by a Novel Isoxazole-Containing Heteroretinoid

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In a search for retinoic acid receptor (RAR and RXR)-selective ligands, a series of isoxazole retinoids was synthesized and evaluated in vitro in transcriptional activation and competition binding assays for RARs and RXRs. In addition, these compounds were evaluated for their differentiating, cytotoxic, and apoptotic activities. In general, these derivatives showed scarcely any binding affinity and were not active in the transcriptional assay. However, among these isoxazole derivatives, the *cis*-isomer **14b** was identified as a potent inducer of apoptosis, and its activity was found to be 6.5 and 4 times superior than that of 13-*cis*- and 9-*cis*-retinoic acids, respectively. On the other hand, compound **13b**, which has the *trans* stereochemistry at the double bond, was found not to be active in the apoptotic assay, but it was endowed with appreciable differentiating activity. Therefore, it seems that the different stereochemistry of the double bond may be associated with a different biological activity: potent apoptotic activity for the *cis*-isomer but differentiating activity for the *trans* structure. This biological behavior was found, at least in part, for the 9-*cis*- and 13-*cis*-retinoic acids with respect to the *all-trans*-retinoic acid. Thus, structure **14b** could offer an interesting model for the design of new compounds endowed with apoptotic activity.

Introduction

Programmed cell death, or apoptosis, is a genetically encoded process involved in the homeostasis of multicellular organisms and in carcinogenesis.[1,2] Several cytotoxic drugs employed in the chemotherapy of malignancies cause apoptosis in neoplastic cells,[3] but the mechanisms by which these drugs induce apoptosis are not well-understood at the molecular level, although several studies indicate that wild-type p53 oncosuppressor gene, the Fas/Fas ligand system, and the caspases activation can play a role in drug-induced apoptosis.[4−6] Recently, it has been shown that some natural and synthetic retinoids are capable of inducing apoptosis in cancer cell lines, but the mechanism by which retinoids induce apoptosis is still unknown.[7−9] The retinoid signal is mediated by two classes of nuclear receptors: the retinoic acid receptors (RARα, -β, and -γ) and the retinoic X receptors (RXRα, -β, and -γ).[10] These receptors usually bind as heterodimers to specific DNA sequences and/or interact with other transcriptional regulators, such as AP-1, to regulate gene transcription. The implication of RARs and RXRs in retinoid-induced apoptosis is still unclear; some observations suggest that cell differentiation mediated by retinoic acid is induced by the activation of RAR, whereas activation of RXR seems to be essential for driving cells into apoptosis;[11] on the contrary, other authors have demonstrated the involvement of the RAR in apoptosis induced by retinoids as shown by the inibition of apoptosis after treatment of HL-60 cells with an RARα antagonist.[12,13] Moreover, a novel retinoid has been recently described that induces apoptosis with mechanism(s) not involving the nuclear RAR.[14]

Thus, as a result of the multiplicity of receptors, hormones, and dimerization pathways, it is likely that the many biological effects associated with retinoids are in fact mediated by several distinct pathways. However, the wider use of retinoids in dermatology (principally in the therapy of psoriasis and acne)[10,15,16] and in other diseases such as oncology (treatment of carcinomas and for cancer chemoprevention)[17,18] has been precluded by unacceptable side effects[19] including skin irritation, lipid and bone toxicity, visual effects, and teratogenicity.[7,19]

To increase the selectivity to the retinoid receptors and to obtain compounds of pharmacological interest, the relationships between structure and retinoid activity have been extensively studied by preparing a large number of geometric isomers as well as conformationally locked and/or restricted analogues, resulting in specific, rigid, three-dimensional configurations.[10,19]

Taking these considerations into account, we have recently started a study aimed at evaluating the substitution of the benzene ring portion of potent retinoids, such as the so-called "short retinoids" TTNPB and AM580, with a more hydrophilic isosteric heterocycle (i.e. isoxazole or thiophene). This study has identified a new class of interesting isoxazole-containing heter-

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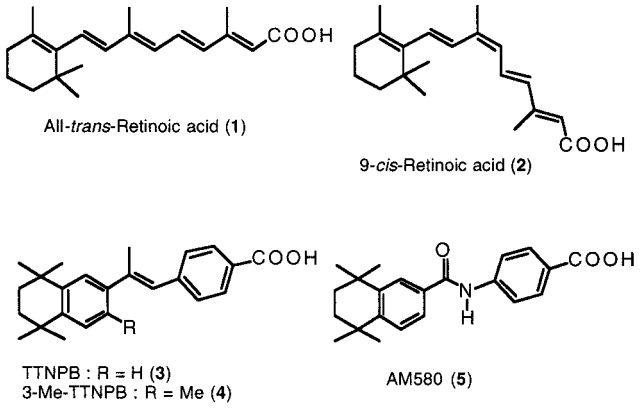
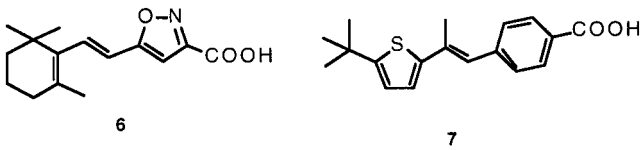
oretinoids and a class of new retinoic acid conjugates.[20−23] Among the isoxazole heteroretinoids some compounds were found to be endowed with significant differentiating and antiproliferative activities; moreover, some new retinoic acid conjugates proved of interest for their antitumor activity and low toxicity.[22]



Natural and synthetic retinoids

In an effort to learn more about the general structure−activity relationships of vitamin A and to better elucidate those structural features that are important to get selectivity toward the different retinoid receptor subfamilies (RARα, -β, and -γ) and with the aim of synthesizing compounds endowed with apoptotic activity, we decided to prepare novel heteroretinoids containing an isoxazole heterocycle in their skeleton. The new compounds were designed by combining features of potent retinoids **3**−**5** with features of some heteroretinoids (compounds **6** and **7** as examples) prepared during our previous studies.[20−23] Briefly, we designed compounds bearing a tetramethyltetrahydronaphthyl group coupled directly (or via an alkenyl portion) to an isoxazole residue.



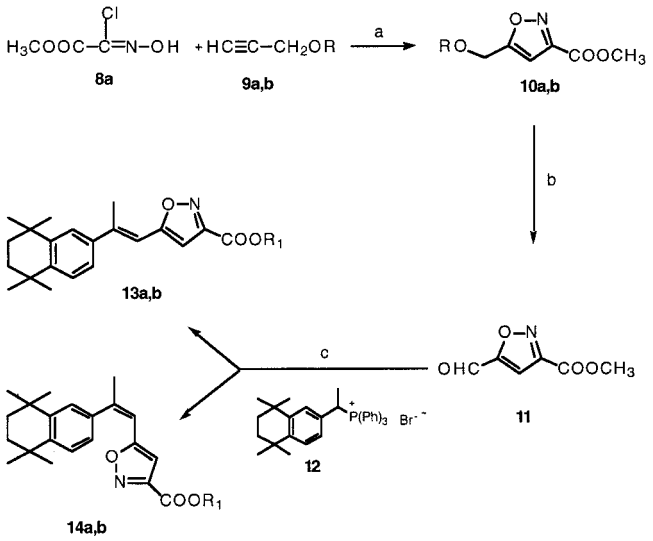
Synthetic Heteroretinoids

Here, we describe our results from the synthesis and the biological activity of some new isoxazole-containing heteroretinoids. All new heteroretinoids **13b**, **14b**, **17**, **18b**, **19b**, and **24** were tested for their differentiating, cytotoxic, and apoptotic activities. The newly synthesized compounds were evaluated also in vitro in transcriptional activation and competition binding assays for RARs and RXRs. In particular, we describe for the first time a novel heteroretinoid (**14b)** containing an isoxazole moiety able to induce apoptosis in HL-60 cells after only 24 h of treatment. We have compared the activity of **14b** with that of three well-characterized retinoids: 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, and *all-trans*-retinoic acid (ATRA).

Chemistry

For the construction of the two heteroretinoids **13b** and **14b** (Scheme 1) our approach started from the

Scheme 1[a]



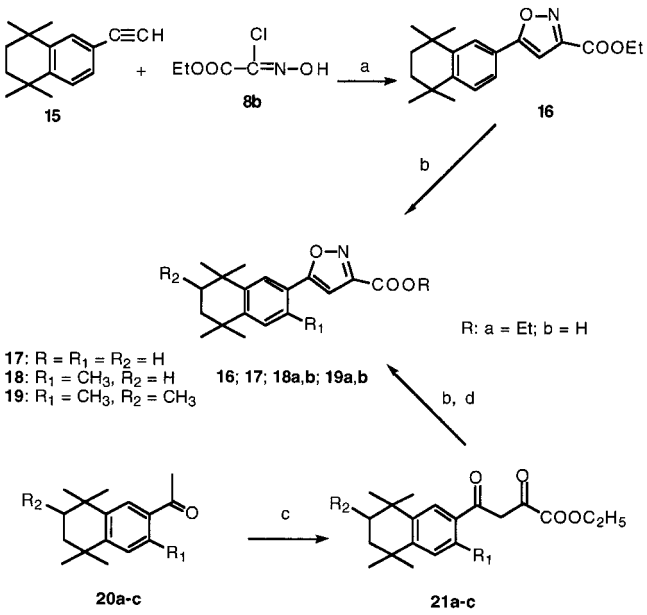
R: a = H, b = Tetrahydro-2*H*-pyran-2-yl
R_1 : a = CH_3, b = H

[a] (a) (i) K_2CO_3, $CHCl_3$, (ii) TEA, Et_2O; (b) PCC, CH_2Cl_2; (c) (i) potassium *tert*-butoxide, DMSO, (ii) LiOH × H_2O, MeOH.

chloro oxime **8a** easily prepared from commercially available glycine methyl ester as described in the literature.[24,25] The nitrile oxide generated from **8a** by treatment with triethylamine was reacted through a [3 + 2] cycloaddition reaction into the alkyne **9b**, to afford in a regioselective manner and good yield the corresponding isoxazole derivative **10b**. Removal of the protective pyranyl group by use of Amberlite H-15 in methanol at 50 °C led to the primary alcohol **10a** in very high yield. The latter derivative has been previously obtained through the [3 + 2] cycloaddition of the nitrile oxide generated from the chloro oxime **8a** into the alkyne **9a** bearing the free alcoholic function.[25] However, in our hands this reaction afforded only a moderate yield of the desired isoxazole. The oxidation of the primary alcohol of **10a** to the aldehyde **11** was easily performed by pyridinium chlorocromate (PCC) in methylene chloride solution in 85% yield. Finally, the Wittig reaction between the aldehyde **11** and the phosphonium salt **12**, which was in turn prepared as described in the literature,[26] afforded in moderate yield a 9/1 mixture of the two isomers **13a** and **14a**. The stereochemistry *E* or *Z* for the latter derivatives was assessed by means of their ^{1}HNMR spectra. The signal for the vinylic proton of the *Z*-isomer appeared at higher field than that of the *E*-isomer. Moreover, the vinylic proton of the *Z*-isomer appears as a doublet with a higher coupling constant. These data agree with those reported for a trisubstituted double bond bearing a methyl group and two aryl functions as substituents; moreover they are consistent with those of other arotinoids previously described in the literature.[26−28] Our data are also corroborated by NOESY and COSY-2D experiments. Finally, the hydrolysis of the ester function by use of lithium hydroxide afforded in good yields the desired **13b** and **14b**.

The preparation of the isoxazole derivatives **17**, **18b**, and **19b** was initially attempted by the cycloaddition of the nitrile oxide generated from the chloro oxime **8b** into the alkyne **15** (Scheme 2). Surprisingly, the reaction

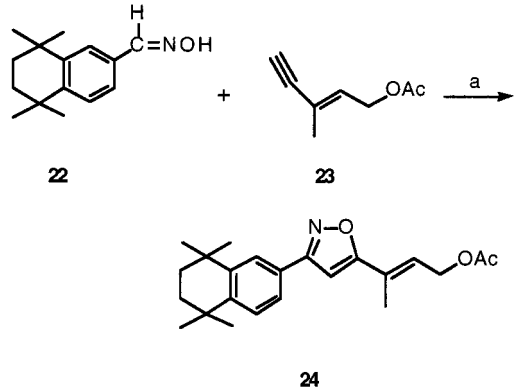
Scheme 2[a]



[a] (a) K_2CO_3, $CHCl_3$; (b) LiOH × H_2O; (c) $(COOEt)_2$, NaOEt, EtOH; (d) NH_2OH × HCl, EtOH, reflux.

afforded only low yield of the desired isoxazole **16**; thus an alternative approach was devised starting from the methyl ketones **20a**−**c**. The ethoxyoxalyl derivatives **21a**−**c** were obtained starting from the methyl ketones **20a**−**c** and diethyl oxalate in the presence of EtONa at room temperature. The latter derivatives were reacted regiospecifically with an excess of hydroxylamine hydrochloride in ethanol solution to afford in good yields the title isoxazoles. Upon treatment with lithium hydroxide in aqueous methanol solution, **16**, **18a**, and **19a** underwent facile hydrolysis of the ester function to give the expected acids **17**, **18b**, and **19b**.

Scheme 3[a]



[a] (a) NCS, Py, TEA, $CHCl_3$.

Finally, the heteroretinoid derivative **24** (Scheme 3) was prepared using chemistry described above: the cycloaddition reaction of the nitrile oxide generated from the oxime **22** into the enyne **23** afforded in chemo- and regioselective manner the desired isoxazole **24**.

Biological Results and Discussion

The retinoids described in this paper were prepared with the aim to discover new lead compounds with clinical potential. In particular, we focused our attention on the indentification of new structural features for the apoptotic activity. The premises for our work on these new heteroretinoids were formulated by preliminary studies coming from our laboratory in which we demonstrated that some new isoxazole-containing heteroretinoids are endowed with differentiating properties against HL-60 cells.[21,23] We have also identified a class of new retinoic acid conjugates proved of great interest for their low toxicity and/or their activity.[23]

Table 1. Transcriptional Activation and Binding Assays for Retinoid Analogues[a]

	RXR[b] EC_{50} (nM) K_d (nM)			RAR[b] EC_{50} (nM) K_d (nM)		
agent	α	β	γ	α	β	γ
	NA			5.0	1.5	0.5
ATRA				16	7	3
	13.0			102	3.3	6.0
9-*cis*-RA						
	NA	NA	NA	NA	>1K	>1K
18b	5.5K	NT	8.1K	9K	>30K	>30K
	NA	NA	NA	NA	>1K	>1K
14b	4.2K	NT	>10K	6K	>30K	7K
	>1K	NA	>1K	NA	>1K	>1K
13b	2.1K	NT	1.4K	1K	>30K	2K
	NA	NA	NA	NA	>1K	>1K
19b	>10K	NT	>100K	5K	>30K	6.1K
	NA	NA	NA	NA	>1K	>1K
17	5.1K	NT	>100K	28K	21K	8.8K

[a] These data were obtained as described in ref 33. [b] For each agent, first row indicates values of transcriptional activation assays and second row indicates values of binding assays. NA, not active; NT, not tested; K, 1000 nM.

Compounds **13b**, **14b**, **17**, **18b**, and **19b** were evaluated in binding assays for RARs and RXRs. The binding for this series of five compounds is presented in Table 1. It is evident from these data that the introduction of the isoxazole heterocycle leads to a marked reduction, as compared with the known retinoids, in affinity at the retinoid receptors. This was somewhat surprising to us, since the potent activity of other short retinoids is well-known. Moreover, compounds structurally related to our derivatives, but containing the thiazole heterocycle, were found to be potent binders of the retinoid receptors. However, the desmethyl analogue **17** has reduced binding data in RARβ and RXRγ with respect to the methyl derivative **18b**, and this is consistent with what was described for TTNPB (**1**) and 3-methyl-TTNPB (**2**). Introduction of one more methyl substituent in the tetramethyltetrahydronaphthyl portion reduces, apart from RARγ, the binding affinity of the compound. Nevertheless, the introduction of the alkenyl portion in compounds **13b** and **14b** increases the binding affinity. The interpretation of these data is not straightforward, but we may advance the hypothesis that probably, at least for this class of compounds, a more structural resemblance to natural retinoids is fundamental for binding activity. In this regard, it is significant that the results of the transcriptional activation assay of these heteroretinoids (Table 1) were higher concentrations than those showed by the *trans*-retinoic acid.

Since retinoids induce HL-60 cells (promyelocytic cell line) to differentiate, the retinoids reported herein were evaluated in the HL-60 assay, using *all-trans*-retinoic acid (ATRA) as the standard (Table 2). Exposure of HL-60 cells for 6 days to a 5×10^{-5} M solution of retinoids **17**, **18b**, **19b**, and **24** resulted in partial differentiation

Table 2. Cytostatic and Differentiating Activity of Compounds **13b**, **17**, **18b**, **19b**, and **24** on HL-60 Cells

compd	cytostatic activity IC_{50} (μM)[a]	cellular differentiation[b] CD14[c] (%)	CD11c[d] (%)	CD11b[d] (%)	morphology
control		0.2	0	0	myeloblasts
DMSO (5%)		0	0	0	myeloblasts
retinoic acid	11 ± 3	0	97.3	98.2	granulocytes
13b	74 ± 8	82.6	12.2	13	monocytes
17	75 ± 11	15.8	0	0	monocytes
18b	72 ± 7	47.23	0	0	monocytes
19b	73 ± 12	20.8	0.4	0	monocytes
24	55 ± 6	30.6	0	0	monocytes

[a] 50% Inhibitory concentration (evaluation after 72 h of drug exposure). [b] Evaluation after 6 days of exposure to each retinoid used at a concentration of 5×10^{-5} M. [c] Monocyte-associated antigen. [d] Granulocyte associated antigen.

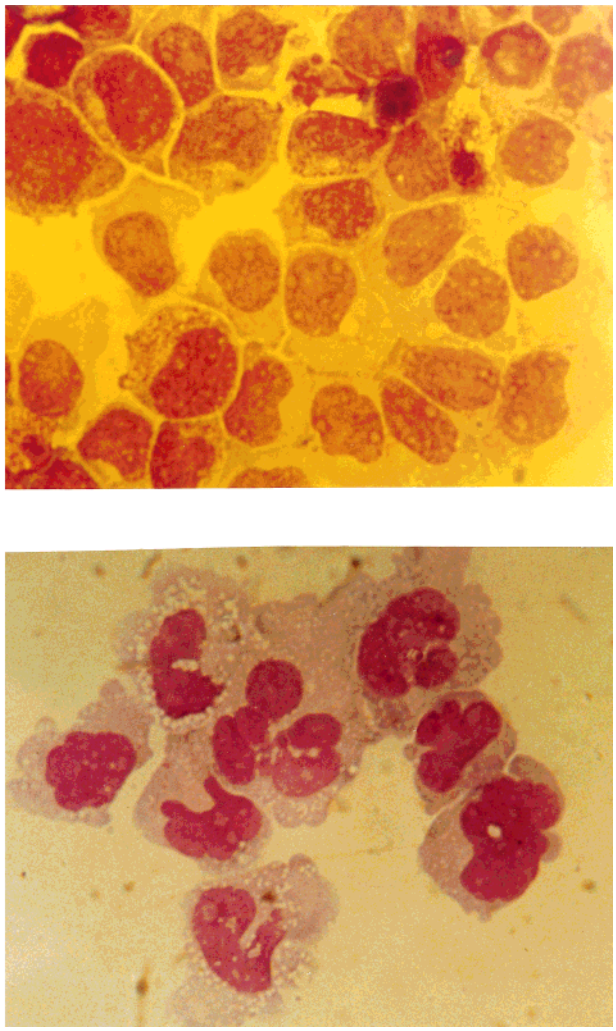


Figure 1. Morphologic changes observed in HL-60 cells after 6 days of exposure to 5×10^{-5} M **13b**: (a) untreated HL-60 cells; (b) treated HL-60 cells. These cells showed a morphology typical of monocytes. Cytospin slide preparation stained with May Grunwald-Giemsa (1000×). (Figure reproduced at 70% of original.)

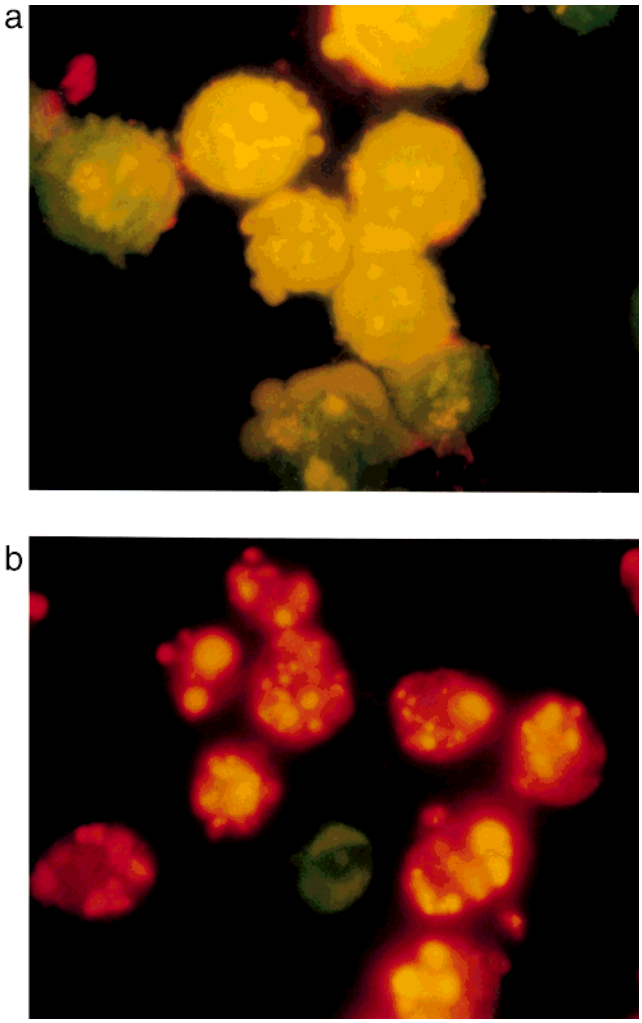


Figure 2. Morphologic changes observed in HL-60 cells after 48 h of exposure to 5×10^{-5} M **14b**. (b) Cells treated with **14b** showed a typical nuclear fragmentation in apoptotic bodies; (a) untreated cells. Evaluation with fluorescence microscopy (1000×) after staining with acridine orange and ethidium bromide. (Figure reproduced at 70% of original.)

toward mature monocytes, while **13b** was able to induce monocytic differentiation in almost all treated cells (Table 1 and Figure 1). No granulocytic differentiation was observed with these heteroretinoids, while the exposure of HL-60 to ATRA for 6 days at a concentration of 5×10^{-5} M induced a complete differentiation into mature granulocytes. In contrast to *trans*-retinoic acid that was active also at a concentration of 5×10^{-8} M, **13b**, **18b**, and **24** were unable to induce differentiation when used at concentrations lower than 1×10^{-6} M.

The analysis of cellular differentiation with monoclonal antibody confirmed the results obtained by morphological assay. All heteroretinoids induced an increase of CD14 expression (monocytic marker), but the expression of CD11c and CD11b (granulocytic markers) was similar to the untreated control. On the contrary, the exposure of HL-60 cells to ATRA induced a marked expression of CD11c and CD11b. Interestingly, the differentiating activity of derivative **13b** was higher than that shown by compounds **17**, **18b**, **19b**, and **24**, thus confirming

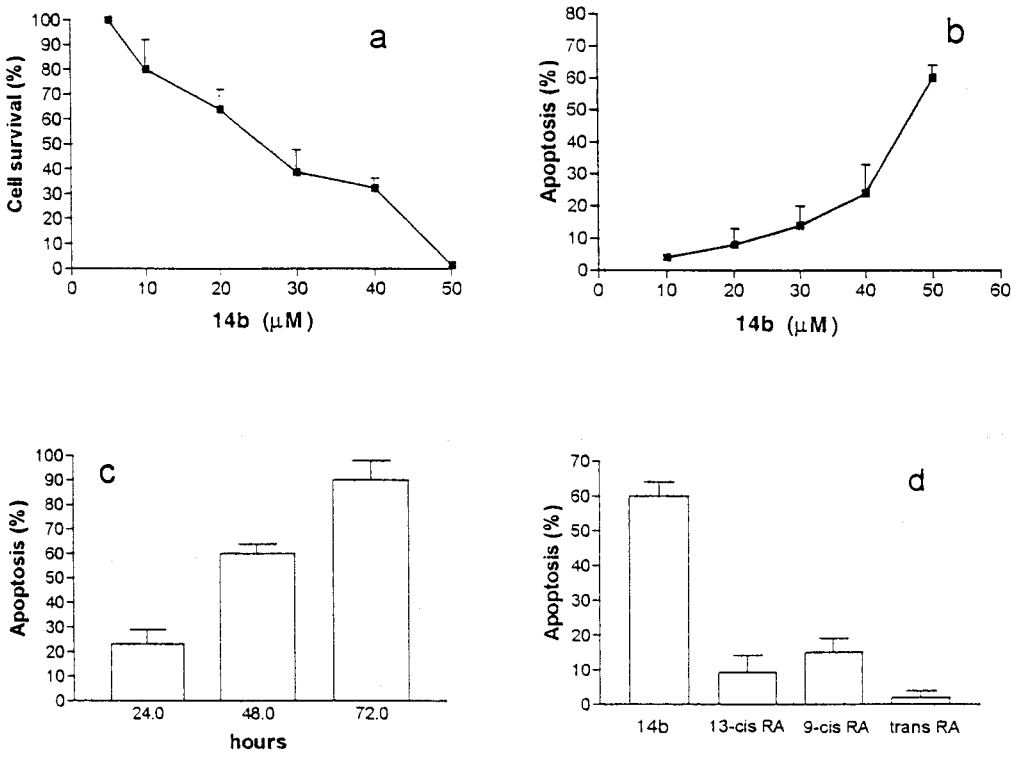


Figure 3. Cytotoxic and apoptotic effects of retinoid **14b** on HL-60 cells: (a) cytotoxicity of **14b** used at different concentrations on HL-60 cells after 48 h of treatment; (b) percentage of apoptotic cells induced by different concentrations of **14b** (evaluation after 48 h of treatment); (c) percentage of apoptotic cells induced by 5×10^{-5} M **14b** after 24, 48, and 72 h of treatment; (d) apoptotic effects of **14b** in comparison with 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, and *trans*-retinoic acid (evaluation after 48 h of treatment with 5×10^{-5} M of each retinoid).

the importance of some structural features, namely the tetramethyltetrahydronaphthyl and the aromatic (a benzene ring or heterocycle) groups linked together by an alkenyl portion.

Retinoic acid treatment of HL-60 cells over a period of 6−8 days results in a progressive increase in the proportion of cells with mature neutrophil morphology and is followed by an increase in the proportion of cells exhibiting the morphological characteristics of apoptosis.[29] Our experiments showed that the treatment of HL-60 cells for 3 days with compounds **13b**, **17**, **18b**, **19b**, and **24** induced a cell growth inhibition (cytostatic effect) but not apoptosis. However, after 6 days of treatment with these compounds, it was evident that a low percentage of apoptotic cells increased in the following days in a manner similar to that observed with *trans*-retinoic acid. This was not observed using compounds **17** and **19b**. Of great interest, the derivative **14b**, bearing the alkenyl portion with the *cis* stereochemistry, thus resembling the known natural 9-*cis*-retinoic acid, was able to induce apoptosis after only 24 h of treatment as shown by morphological and flow cytometric assays (Figures 2 and 4). Figure 3a,b shows the cytotoxic and apoptotic effect of **14b** on HL-60 cells after 48 h of treatment and Figure 3c the apoptotic activity of **14b** at a concentration of 5×10^{-5} M after 24, 48, and 72 h. No cell differentiation was observed in HL-60 cells treated with **14b** probably because of the quick induction of apoptosis by this compound.[30]

The apoptosis-inducing activity of **14b** was 6.5 and 4 times higher than that of 13-*cis*-retinoic acid and 9-*cis*-retinoic acid, respectively, while *trans*-retinoic acid was unable to induce apoptosis (evaluation after 48 h of treatment) (Figure 3d). Flow cytometry assay performed after 24 h of exposure of HL-60 cells to different concentrations of **14b** (20, 30, 40, and 50 mM) showed a reduction of cell distribution in the S-G2M phase of cell cycle with a slight cell recruitment in the G1 phase with a sub-G1 apoptotic peak (Figure 4). Moreover, morphological assay of HL-60 cells treated with **14b** did not show typical figures of necrosis.[32] Interestingly, compounds **13b** and **14b**, having as a unique difference the stereochemistry of the double bond, possess different apoptotic and differentiating activities: the different stereochemistry seems to be associated with a different biological activity (quick and marked apoptotic activity for **14b**, differentiating activity for **13b**). This different biological behavior was also observed, at least in part, by treating HL-60 cells with 9-*cis*- or 13-*cis*-retinoic acid (prevalent apoptosis) or with *trans*-retinoic acid (prevalent differentiation).

To determine whether **14b** was able to induce cytotoxicity in tumor cells resistant to conventional chemotherapeutic drugs, we tested this retinoid in a multidrug-resistant variant of the HL-60 cell line (HL-60R) expressing high levels of P-glycoprotein (Pgp), a cell membrane glycoprotein able to pump out of cells toxic and chemotherapeutic agents. We observed that HL-60R cells were completely resistant to the cytotoxic activity of **14b**. This resistance was unrelated with Pgp expression; in fact, using drugs able to block the drug-efflux activity of Pgp, such as verapamil (5 μg/mL) or cyclosporin (2 μg/mL), we did not observe any increase of cell growth inhibition (data not shown), thus excluding an

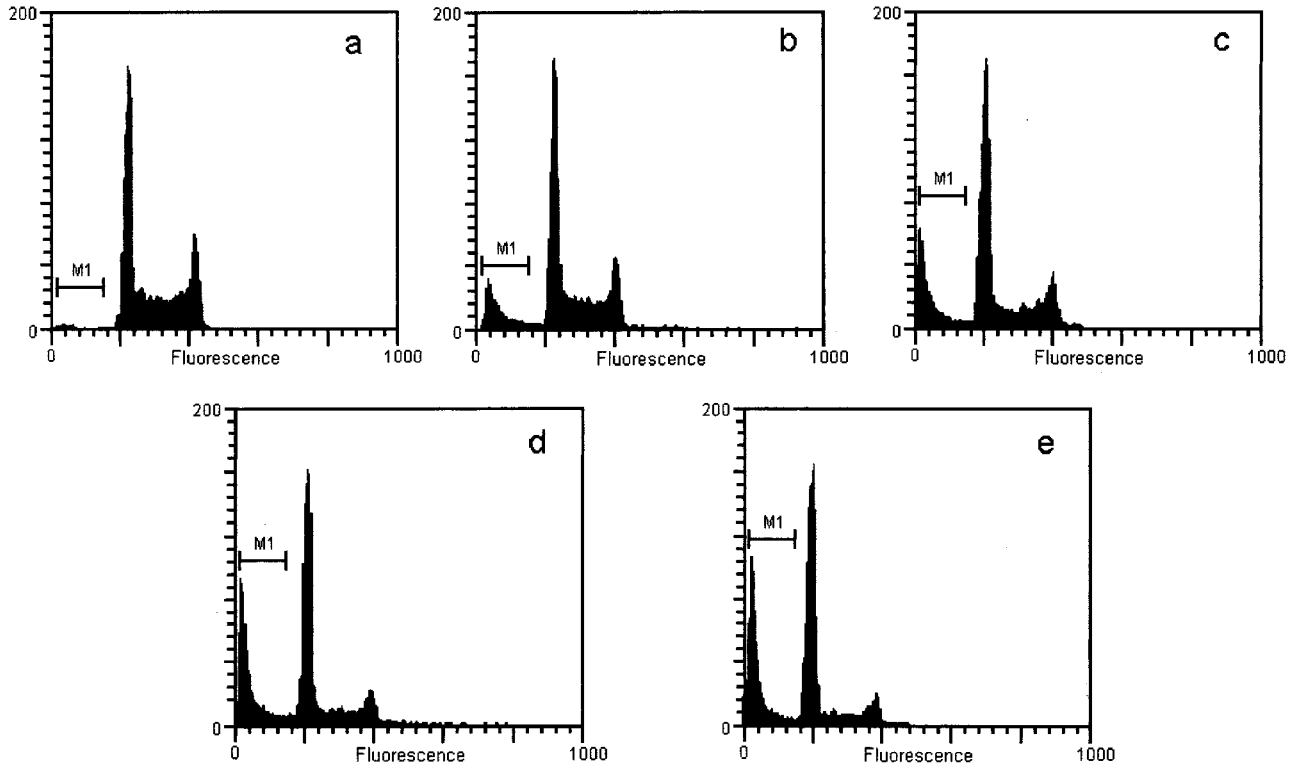


Figure 4. Cell cycle distribution of HL-60 cells treated 24 h with retinoid **14b** used at a concentration of 20 μM (b), 30 μM (c), 40 μM (d), and 50 μM (e); (a) untreated HL-60 cells. M1 = apoptotic sub-G1 area.

involvement of Pgp in **14b** retinoid resistance. Moreover, we observed that **14b** was inactive in cell lines commonly resistant to retinoic acids such as the K562 cell line.

In conclusion, we have found for the first time a new isoxazole heteroretinoid (**14b**) endowed with potent apoptotic activity; the apoptosis-inducing activity of this compound was higher than that of other known retinoic acid derivatives (9-*cis*- and 13-*cis*-retinoic acid, *all-trans*-retinoic acid). To the best of our knowledge, **14b** represents the first example of a heteroretinoid endowed with potent apoptotic activity. Therefore, due to its marked apoptotic activity, **14b** may represent an interesting compound in the treatment of acute promyelocytic leukemia. In addition, in our experiments we confirmed the observations of other authors that retinoic acid isomers can induce HL-60 cells to undergo apoptosis; in particular, 9-*cis*-retinoic acid (well-known as a potent apoptosis inducer)[8] showed potent apoptotic activity also in our test. Since it has been postulated that the apoptotic activity of 9-*cis*-retinoic acid may be due to its ability to activate both RARs and RXRs and our compounds have scarcely any binding affinity (see Table 1: 2.1K → 100K), it is likely that a different unknown mechanism may be involved. We are currently investigating the possibility of using the structure of **14b** as a model in designing new compounds endowed with apoptotic activity.

Experimental Section

General Methods and Materials. Melting points were obtained in open capillary tubes and are uncorrected. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F_{254} Merck plates. Infrared spectra (IR) were measured on a Perkin-Elmer 257 instrument. Nuclear magnetic resonance (^{1}H NMR) spectra were determined in $CDCl_3$ solution with a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as internal standard. Petroleum ether refers to the fractions boiling in the range 40−60 °C. 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 with calculated values within ±0.4% of the theoretical values.

Methyl 5-(Tetrahydro-2*H*-pyran-2-yloxymethyl)isoxazole-3-carboxylate (10b). To a stirred solution of methyl chlorooximidoacetate (**8a**) (4.8 g, 35 mmol) and the alkyne **9b** (14.7 g, 105 mmol) in 100 mL of dry ether was slowly added a solution of triethylamine (35 mmol) in 50 mL of diethyl ether (30 min). The reaction was allowed to stir at room temperature for 12 h. The mixture was washed with water, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on silica gel (eluent: petroleum ether/EtOAc, 8:2): 8 g, 95% yield, oil; IR (neat) 1740, 1610 cm^{-1}; ^{1}H NMR δ 1.5−1.8 (m, 6H); 3.55 (m, 1H); 3.84 (m, 1H); 3.97 (s, 3H); 4.63−4.86 (m, 3H); 6.66 (s, 1H). Anal. ($C_{11}H_{15}NO_5$) C, H, N.

Methyl 5-Hydroxymethylisoxazole-3-carboxylate (10a). The isoxazole derivative **10a** was attempted also as described in the literature[24,25] from the alkyne **9a** but with low yield (15−30%). Better results were obtained starting from the alkyne **9b** as described above.

The tetrahydropyranyl derivative **10b** (7 g, 30 mmol) was dissolved in MeOH (50 mL) and stirred in the presence of Amberlite H-15 (500 mg) at 50 °C for 5 h. Filtration and removal of the solvent under reduced pressure left a crude oil which was flash chromatographed on silica gel (eluent: petroleum ether/EtOAc, 7:3): 4.15 g, 88% yield, oil; IR (neat) 3300, 1740, 1610 cm^{-1}; ^{1}H NMR δ 3.5 (br, 1H); 3.98 (s, 3H); 4.83 (s, 2H); 6.66 (s, 1H). Anal. ($C_9H_7NO_4$) C, H, N.

Methyl 5-Formylisoxazole-3-carboxylate (11). The alcohol **10a** (0.5 g, 3.18 mmol) was dissolved in dichloromethane (50 mL), cooled to 0 °C, and treated with pyridinium chlorochromate (1.37 g, 6.37 mmol). The reaction mixture was stirred

at room temperature for 5 h, diluted with diethyl ether (50 mL), filtered through Celite, and concentrated under reduced pressure. Flash chromatographic purification of the oily residue (diethyl ether/petroleum ether, 1/9) gave the aldehyde **11** as an oil: 330 mg, 67% yield, oil; IR (neat) 1740, 1580 cm^{-1}; ^1H NMR δ 4.02 (s, 3H); 7.37 (s, 1H); 10.14 (s, 1H). Anal. ($C_6H_5NO_4$) C, H, N.

General Procedure for Formation of Isoxazoles 13a and 14a. The phosphonium salt **12** (6.1 g, 11 mmol) was added, at ambient temperature, to a stirred solution of potassium *tert*-butoxide (1.1 g, 10 mmol) in anhydrous dimethyl sulfoxide (10 mL). The mixture was stirred for 1 h until dissolution was complete. The aldehyde **11** (1.3 g, 8.6 mmol) in dimethyl sulfoxide (5 mL) was then added dropwise to the solution. The mixture was stirred at 25 °C for 1 h, then quenched with saturated aqueous sodium chloride (200 mL), and extracted with diethyl ether (2 × 100 mL). The combined ether extracts were washed with saturated aqueous sodium chloride, dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel (eluent: diethyl ether−light petroleum). Compounds **13a** and **14a** were obtained in a 43% total yield (mixture 9/1).

Methyl (*E*)-5-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]isoxazole-3-carboxylate (13a): oil; IR (neat) 1740, 1610, 1600, 1580 cm^{-1}; ^1H NMR δ 1.28 (s, 6H); 1.35 (s, 6H); 1.71 (s, 4H); 2.36 (d, 3H, J = 1.0 Hz); 3.88 (s, 3H); 6.69 (s, 1H); 6.73 (d, 1H, J = 1.0 Hz); 7.35 (m, 2H); 7.45 (s, 1H). Anal. ($C_{22}H_{27}NO_3$) C, H, N.

Methyl (*Z*)-5-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]isoxazole-3-carboxylate (14a): oil; IR (neat) 1740, 1615, 1590, 1580 cm^{-1}; ^1H NMR δ 1.19 (s, 6H); 1.27 (s, 6H); 1.64 (s, 4H); 2.25 (d, 3H, J = 1.87 Hz); 3.87 (s, 3H); 5.65 (s, 1H); 6.45 (d, 1H, J = 1.87 Hz); 6. 99 (d, 1H, J = 8.4 Hz); 7.12 (s, 1H); 7.35 (d, 1H, J = 8.2 Hz). Anal. ($C_{22}H_{27}NO_3$) C, H, N.

General Procedure for Formation of Isoxazoles 16, 18a, and 19a. A mixture of the appropriate methyl ketone **20a**−**c** (46 mmol) and diethyl oxalate (6.7 g, 46 mmol) was added dropwise to an ice-cooled solution of EtONa (1.1 g, 48 mmol, of metallic Na) in absolute EtOH (50 mL). After being allowed to stand overnight at room temperature, the mixture was diluted with water (100 mL), acidified with 10% HCl, and extracted with Et_2O (5 × 35 mL). The combined extracts were washed with brine, dried, and concentrated in vacuo to leave **21a**−**c** as oils which were used in the next step without any further purification. Hydroxylamine hydrochloride (6.1 g, 89 mmol) was added to a solution of the appropriate oxalyl derivative **21a**−**c** (29 mmol) in ethanol (60 mL) and the resulting mixture was heated at reflux conditions for 4 h. The solution was then cooled and concentrated to leave an oil which was poured into water (200 mL) and extracted with Et_2O (3 × 50 mL). The combined organic layers were washed with brine (2 × 50 mL), saturated aqueous sodium hydrogen carbonate (2 × 50 mL), and brine (2 × 50 mL), then dried, and concentrated under reduced pressure. The residual oil was purified by flash chromatography.

Ethyl 5-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)isoxazole-3-carboxylate (16): yield 65%; mp 72−74 °C; IR (KBr) 1740, 1616, 1578 cm^{-1}; ^1H NMR δ 1.30 (s, 6H); 1.33 (s, 6H); 1.45 (t, 3H, J = 7.3 Hz); 1.71 (s, 4H); 4.47 (q, 2H, J = 7.3 Hz); 6.87 (s, 1H); 7.41(d, 1H, J = 8.2 Hz); 7.55 (d, 1H, J = 8.2 Hz); 7. 74 (s, 1H). Anal. ($C_{20}H_{25}NO_3$) C, H, N.

Ethyl 5-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)isoxazole-3-carboxylate (18a): yield 78%; mp 95−96 °C; IR (KBr) 1738, 1615, 1580 cm^{-1}; ^1H NMR δ 1.37 (s, 12H); 1.51 (t, 3H, J = 7.1 Hz); 1.76 (s, 4H); 2.53 (s, 3H); 4.53 (q, 2H, J = 7.1 Hz); 6.66 (s, 1H); 7.32 (s, 1H); 7. 74 (s, 1H). Anal. ($C_{21}H_{27}NO_3$) C, H, N.

Ethyl 5-(5,6,7,8-tetrahydro-3,5,5,7,8,8-hexamethyl-2-naphthalenyl)isoxazole-3-carboxylate (19a): yield 44%; mp 105−108 °C; IR (KBr) 1740, 1618,1580 cm^{-1}; ^1H NMR δ 1.02−1.35 (m, 15H); 1.45 (t, 3H, J = 7.1 Hz); 1.62 (m, 2H); 1.91 (m, 1H); 2.48 (s, 3H); 4.47 (q, 2H, J = 7.1 Hz); 6.79 (s, 1H); 7.26 (s, 1H); 7. 67 (s, 1H). Anal. ($C_{22}H_{29}NO_3$) C, H, N.

General Procedure for Formation of Carboxylic Acids 13b, 14b, 17, 18b, and 19b. A mixture of the appropriate ester (7.7 mmol), methanol (30 mL), water (20 mL), and lithium hydroxide (0.27 g, 11.5 mmol) was allowed to stand at room temperature for 24 h. The solution was concentrated in vacuo to remove methanol and the remaining aqueous solution was extracted with ether to remove trace amounts of unreacted ester. The aqueous solution was acidified with 1 M hydrochloric acid and extracted with three portions of ethyl acetate. 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 crystallized.

5-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-isoxazolecarboxylic acid (17): yield 95%; mp 165−168 °C (EtOAc/hexane); IR (KBr) 3300−2700, 1710, 1610, 1590 cm^{-1}; ^1H NMR δ 1.31 (s, 6H); 1.35 (s, 6H); 1.73 (s, 4H); 6.94 (s, 1H); 7.42 (d, 1H, J = 8.4 Hz); 7.54 (d, 1H, J = 8.4 Hz); 7.77 (s, 1H), 13.8 (br, 1H). Anal. ($C_{18}H_{21}NO_3$) C, H, N.

5-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-3-isoxazolecarboxylic acid (18b): yield 98%; mp 196−197 °C (EtOAc/hexane); IR (KBr) 3300−2700, 1715, 1605, 1590 cm^{-1}; ^1H NMR δ 1.31 (s, 12H); 1.64 (s, 4H); 2.48 (s, 3H); 7.11 (s, 1H); 7.34 (s, 1H); 7. 64 (s, 1H); 13.5 (br, 1H). Anal. ($C_{19}H_{23}NO_3$) C, H, N.

5-(5,6,7,8-Tetrahydro-3,5,5,7,8,8-hexamethyl-2-naphthalenyl)-3-isoxazolecarboxylic acid (19b): yield 95%; mp 203−205 °C (EtOAc/hexane); IR (KBr) 3300−2700, 1710, 1605, 1590 cm^{-1}; ^1H NMR δ 0.85−1.34 (m, 16H); 1.44 (m, 1H); 1.85 (m, 1H); 2.49 (s, 3H); 6.66 (s, 1H); 7.29 (s, 1H); 7.69 (s, 1H); 13.5 (br, 1H). Anal. ($C_{20}H_{25}NO_3$) C, H, N.

(*E*)-5-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-3-isoxazolecarboxylic acid (13b): yield 87%; mp 137−138 °C (Et_2O/pentane); IR (KBr) 3300−2700, 1710, 1610, 1590 cm^{-1}; ^1H NMR δ 1.23 (s, 6H); 1.27 (s, 6H); 1.64 (s, 4H); 2.36 (s, 3H); 6.56 (s, 1H); 6.74 (s, 1H); 7.32 (m, 2H); 7.48 (s, 1H); 13.1 (br, 1H). Anal. ($C_{21}H_{25}NO_3$) C, H, N.

(*Z*)-5-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-3-isoxazolecarboxylic acid (14b): yield 84%; mp 125−127 °C (Et_2O/pentane); IR (KBr) 3300−2700, 1713, 1600, 1590 cm^{-1}; ^1H NMR δ 1.18 (s, 6H); 1.25 (s, 6H); 1.64 (s, 4H); 2.16 (s, 3H); 5.65 (s, 1H); 6.44 (s, 1H); 6. 99 (d, 1H, J = 8.2 Hz); 7.16 (s, 1H); 7.36 (d, 1H, J = 8.2 Hz), 13.1 (br, 1H). Anal. ($C_{21}H_{25}NO_3$) C, H, N.

3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-5-[(*E*)-3-(acetyloxy)-1-methyl-1-propenyl]isoxazole (24). Hydroxylamine hydrochloride (1.6 g, 23.2 mmol) was dissolved at 0 °C in water (10 mL) containing $NaHCO_3$ (1.8 g, 28.8 mmol). The mixture was left at room temperature for 10 min and then the aldehyde (4.5 g, 19.2 mmol) dissolved in methanol (20 mL) was added portionwise. Methanol was evaporated in vacuo and the mixture extracted with diethyl ether. The organic extracts were washed with brine, dried, and evaporated under reduced pressure. The residue was crystallized from diethyl ether−petroleum ether to afford **22**: 4 g, 90% yield; mp 125−126 °C.

The oxime **22** (4.6 g, 20 mmol) was added at 25 °C to a solution of chlorosuccinimide (2.67 g, 20 mmol) in dry chloroform (18 mL) containing 0.1 mL of pyridine. After 20 min at 25 °C the alkyne **23** (3 g, 25 mmol) was added in one portion and then triethylamine (3.48 mL, 25 mmol) dissolved in $CHCl_3$ (3 mL) was added dropwise over 30 min. The mixture was stirred for 1.5 h, washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue was flash chromatographed on silica gel (ethyl acetate/light petroleum, 3/7) to give **24** as an oil (7 g, 67%): IR (neat) 1740, 1580 cm^{-1}; ^1H NMR δ 1.31 (s, 6H); 1.33 (s, 6H); 1.61 (s, 4H); 1.71 (s, 3H); 2.23 (s, 3H); 4.64 (d, 2H, J = 6.5 Hz); 6.45 (tbr, 1H); 6.50 (s, 1H); 7.41 (d, 1H, J = 7.4 Hz); 7.52 (s, 1H); 7.74 (d, 1H, J = 7.4 Hz). Anal. ($C_{23}H_{29}NO_3$) C, H, N.

Transcriptional Activation and Binding Assays. The transcriptional and binding assays were measured essentially as previously described.[33]

Cell Culture. HL-60 and HL-60R cells were grown in RPMI 1640 (Gibco, Grand Island, NY) containing 10% FCS (Gibco), 100 U/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.

Cytotoxicity Assay. To evaluate the number of live and dead cells, cells were stained with trypan blue and counted on a hemocytometer. Cells which showed trypan blue uptake were interpreted as nonviable. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25-mm wells (Costar, Cambridge, U.K.) 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 determined and expressed as percent of control proliferation.

Flow Cytometric Analysis per Cell Cycle and Apoptosis. The cells were washed once in ice-cold PBS and resuspended at 1×10^6 cells/mL in a hypotonic fluorochrome solution containing propidium iodide (Sigma), 50 g/mL in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, and their fluorescence was analyzed as single-parameter frequency histograms using a FACSort (Becton Dickinson, Mountain View, CA). Apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G0-G1 peak after labeling with propidium iodide.

Analysis of Cellular Differentiation. Cells (2×10^5) were exposed to 50 μM of 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 Grünwald-Giemsa. The expression of monocytic (CD14), granulocytic (CD11c, CD11b, CD15), or promyelocytic (CD15) cell surface antigen was studied by the two-color direct immunofluorescence staining technique. Cells were stained using fluorescein isothiocyanate (FITC)-conjugate mouse anti-human CD14 and phycoerythrin-conjugated mouse anti-human CD11c (both from Becton Dickinson, Mountain View, CA) or FITC-conjugate mouse anti-human CD15 (Becton Dickinson) and phycoerythrin-conjugated mouse anti-human CD11b (Becton Dickinson). Control studies were performed with nonbinding control murine IgG1 and IgG2 isotype antibodies (Becton Dickinson). Analysis of fluorescence was performed on a FACScan flow cytometer (Becton Dickinson).

Morphological Evaluation of Apoptosis and Necrosis. Drug effects on 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; 10 μL of the mixture was placed on a microscope slide, covered with a 22-mm^2 coverslip, and examined in an oil immersion with a 100× objective lens using a fluorescent microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. 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.

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