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Programmed Cell Death (PCD) Associated with the Stilbene Motif of Arotinoids: Discovery of Novel Apoptosis Inducer Agents Possessing Activity on Multidrug Resistant Tumor Cells

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Abstract—Considering that the stereochemistry of the C9–C10 alkenyl portion of natural 9-*cis*-RA, as the one of the olefinic moiety of the previously described isoxazole retinoid **4**, seems of particular importance for their apoptotic activity, we prepared a novel class of TTNPB analogues bearing both the *cis* or *trans* configuration of the alkenyl portion. The compounds were evaluated in vitro for their cytotoxic and apoptotic activities. We discovered that the *cis*-TTNPB **9c** possesses apoptotic activity comparable with that of the retinoid **4**. Moreover, the amino arotinoid **16c** showed potent apoptotic activity in HL60 promyelocytic leukemia cells. Interestingly, **16c** proved to be a particularly potent apoptosis-inducing agent active in multidrug resistant (MDR) cell lines. Therefore, to the best of our knowledge, **16c** may represent the first known aminoarotinoid endowed with potent apoptotic activity in MDR cells. Taken together, these results seem to point out that the *cis*-stilbene motif of arotinoids may be at least an important feature in conferring cytotoxic and apoptotic activity to this class of compounds.

Introduction

Retinoids are a class of natural and synthetic compounds structurally related to all-*trans*-retinoic acid (ATRA).[1,2] These biological agents are known to play a major role in regulating growth and differentiation of normal, premalignant, and malignant cell types. Therefore, they are currently being evaluated as preventative or therapeutic agents in a variety of human cancers.[3] It has been shown that retinoids can augment the activity of other biological and chemotherapeutic agents, offering potentially effective combination regimens.[3] Moreover, by inducing apoptosis, these compounds may enhance sensitivity of tumors to cytotoxic agents and overcome drug resistance.[3–5]

Retinoids exert most of their effects by binding to specific receptors and modulating gene expression. The diversity of retinoid-induced signalling pathways is mediated by at least six retinoid receptors which fall into two subfamilies: retinoic acid receptors (RARs), α, β and γ, and the retinoid X receptors (RXRs), α, β and γ.[1] In common with other members of the steroid hormone receptor superfamily, these two subfamilies of receptors contain a DNA-binding domain and a ligand-binding domain united by a short hinge region that may also serve as a nuclear transactivation signal. At the cellular level, activation of the retinoid receptors can inhibit cell proliferation, induce differentiation and apoptosis; the induction of apoptosis is related to cell growth and differentiation in various ways, depending on the cell type. The relative implication of RAR and RXR receptors in retinoid-induced apoptosis is still unclear; some observations suggest that cell differentiation mediated by retinoic acid is induced by the activation

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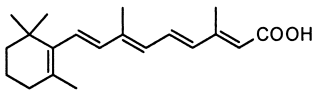
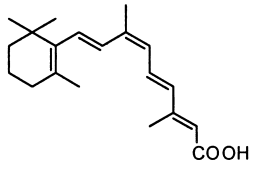
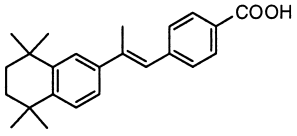
of RARs, whereas activation of RXRs seems to be essential for driving cells into apoptosis;[6] on the contrary, other authors have demonstrated the involvement of the retinoic acid receptor in apoptosis induced by retinoids, as shown by the inhibition of apoptosis after treatment of HL60 cells, with a RARα antagonist.[7,8] However, although the mechanisms of retinoid-induced apoptosis still remain unclear, the clinical efficacy of most of the novel potent retinoids, i.e. 9-*cis*-retinoic acid (9-*cis*-RA),[9] CD437,[10] LDG1069 and *N*-(4-hydroxyphenyl)-retinamide (4-HPR)[11] seems to be related to both their differentiation- and apoptosis-inducing activity.[12]



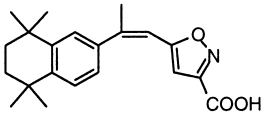
All-*trans*-Retinoic acid (ATRA) **(1)**



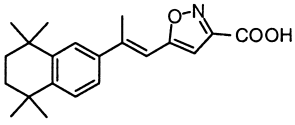
9-*cis*-Retinoic acid (9-*cis*-RA)**(2)**



TTNPB **(3)**



4



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Since 9-*cis*-RA is well known to possess both apoptosis- and differentiation-inducing activities, whereas ATRA induces differentiation but not apoptosis, we considered that the 9-*cis* configuration contained in the polyene side chain of 9-*cis*-RA, as well as the *cis* configuration of the olefin contained in **4**, may be related to the apoptotic activity of these compounds. Consequently, we planned the synthesis of novel TTNPB-retinoid analogues taking into consideration both the configuration *E* and *Z* of the stilbene motif. Thus, we discovered that the *cis*-TTNPB **9c**, which still retains some structural features of 9-*cis*-RA, was endowed with a potent apoptotic activity, being more active than the previously described isoxazole retinoid **4**. On the other hand, TTNPB **3**, a known potent differentiating arotinoid, is only a poor inducer of apoptosis. We found also that the amino derivative **16c** proved to be a particularly potent apoptosis-inducing agent active in multidrug resistant (MDR) cell lines, in particular resistant to the apoptotic effects of several chemotherapeutic drugs, such as daunorubicin, methotrexate, citarabine, 5-fluorouracil and others.

On the other hand, considering that apoptosis is probably the major modality by which leukemic cells are killed by treatment with pharmacological agents, it is likely that the identification of new retinoids able to induce apoptosis in different types of tumor cells, independently/or not of their differentiating activity, could be an important goal in the cancer chemotherapy. Taking these considerations into account, we started a study aimed to design retinoid compounds endowed with potent apoptotic activity.[13–15] We focused our attention on the identification of the structural features associated with the apoptotic activity of compounds related to arotinoids. We took advantage of our previous work on a new class of isoxazole-containing arotinoids, which were found to possess interesting apoptotic activity.[15] In particular, we have reported recently on the effects of the substitution of the phenyl ring of the stilbene skeleton of TTNPB with an isoxazole heterocycle: while the *E* isomer **5** was found to be endowed with an appreciable differentiating activity, the *cis*-stilbene derivative **4** was essentially inactive at the level of the retinoid receptors, but was identified as a potent inducer of apoptosis. These facts led us to hypothesize the existence of a non-RAR-mediated mechanism associated with the *cis*-stilbene-like motif of **4** and responsible for the apoptotic activity of the compound. On the other hand, although 4-HPR and CD437 activate nuclear receptors, their pro-apoptotic activity appears to be mediated, in part, through retinoid receptor-independent pathways.[10,11] Interestingly, 4-HPR and CD437 proved very active in multidrug resistant tumor cells.[16–18]

In our opinion these findings may provide an important basis to facilitate the design of novel potent apoptosis-inducing agents structurally related to the *cis*-stilbene motif of arotinoids. The newly synthesized retinoids will also be important in studies of receptor selectivity aimed to better clarify the mechanism of action of vitamin A and of its analogues (retinoids).

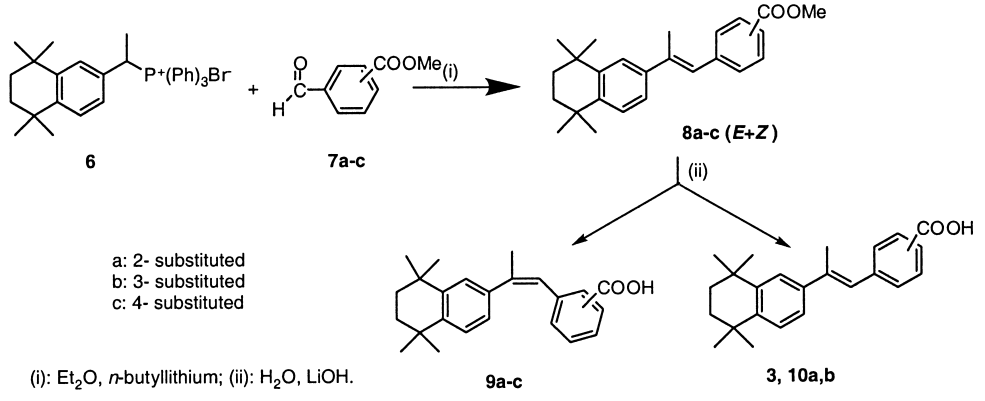
Chemistry

For the procurement of the benzoic acid derivatives **3**, **9a**–**c**, **10a**,**b** we took advantage (Scheme 1), to some extent, of known procedures:[19] the Wittig reaction between the carbomethoxybenzaldehydes **7a**–**c** and the phosphonium salt **6** in the presence of *n*-butyllithium produced the esters **8a**–**c** in significant yields. The reaction produced a mixture of the *E* and *Z* isomers (7:3) which was in turn easily hydrolyzed with lithium hydroxide and the desired carboxylic acid derivatives purified and separated by flash chromatography.

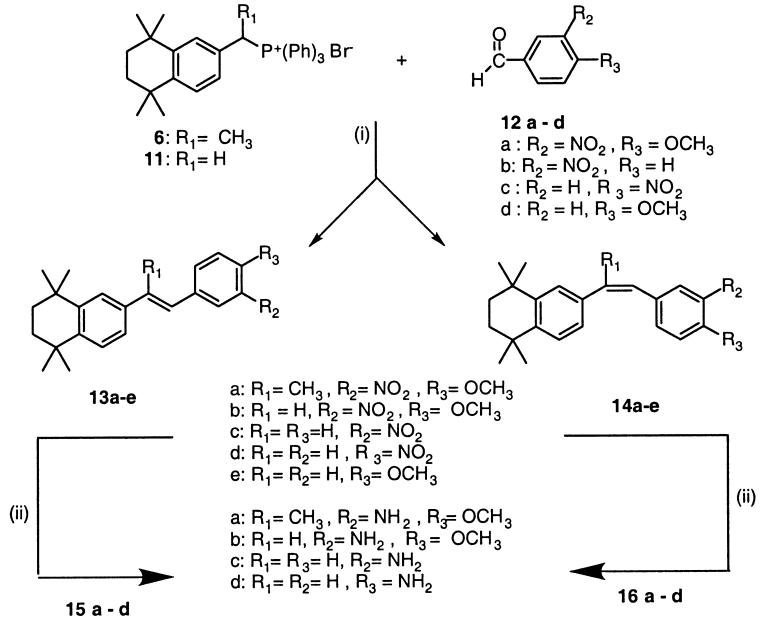
Spectral data of known and new compounds are consistent with those of arotinoids previously described in the literature.[19–21] Finally, the Wittig reaction between the phosphonium salts **6** and **11** and the aldehydes **12a–d** in THF solution and in the presence of NaH as the base produced in good yields the compounds **13a–e** and **14a–e** (Scheme 2). The *E* and *Z* isomers were separated and characterized; bond geometry of the compounds was established by comparison of the ^{1}H NMR of the isomeric pairs. *Z* isomers showed the olefinic protons 0.3–0.45 ppm higher field than those of the *E* isomers. The coupling constants of the vinylic protons of the *E* isomers **13a–e** were about 16 Hz whereas the *Z* isomers **14a–e** showed the coupling constants of 12 Hz. The compounds **13a–d** and **14a–d** were reduced with Zn/AcOH at room temperature to give the amine derivatives **15a–d** and **16a–d** in about 80% yields.

Results and Discussion

Table 1 shows the cytotoxic activity expressed as IC_{50} (the drug concentration able to inhibit by 50% cell growth) and apoptotic activity expressed as AC_{50} (the drug concentration able to induce apoptosis in 50% of cells) of 16 different new retinoid derivatives in the HL60 promyelocytic leukemia cell line. On the basis of their effects on HL60 cells these compounds could be classified into four groups: (1) compounds with a modest cytotoxic activity in terms of cell growth inhibition and unable to activate programmed cell death (**3**, **13e** and **14e**); (2) compounds moderately effective in inducing cytotoxic and apoptotic effects (**9a**,**b**); (3) highly cytotoxic compounds endowed with a moderate apoptotic activity (**9c**, **15a**, **15b**, **15c**, **15d**, **16b** and **16d**); and (4) compounds endowed with both a high cytotoxic and



Scheme 1.



Scheme 2.

apoptotic activity (**16c**). Derivatives **10a**,**b** were devoid of any activity.

Previously, we observed that isoxazole arotinoids with the *cis* stereochemistry were more efficient inducers of apoptosis than their correspondent *trans* stereoisomers. Here we observed that the *cis* isomer of the potent differentiating agent TTNPB is able to induce apoptosis in HL60 cells, while the *trans* isomer has only moderate effects in inhibiting cell proliferation. This was not observed in the case of the *trans* isomer of **15a**, which is twice as active in inducing programmed cell death than the respective *cis* isomer **16a**. However, at difference, in the case of the most active compound **16c**, the *cis* isomer is clearly the most apoptosis-inducing agent. Therefore, although we hypothesized that the different stereochemistry of the double bond may be associated with a different apoptotic activity, this can not be considered an absolute requisite. However, it appears evident from the data shown in Table 1 that a relation exists between apoptosis and the *cis* configuration of the alkenyl portion. Therefore, the *cis*-stilbene motif of arotinoids seems to be at least an important feature in conferring cytotoxic and apoptotic activity to this class of retinoids. Moreover, it also appears evident that the substituent's position at the phenyl portion of the TTNPB analogues is a crucial factor for their activity.

Table 1. Cytotoxic and apoptotic activity of different retinoid derivatives in HL60 cells

Compound no.	IC_{50} (μM)	AC_{50} (μM)[a]
3	46 (±6.2)	>100
9a	52 (±7.3)	90 (±7.8)
9b	50 (±10)	80 (±9.8)
9c	10 (±2.8)	40 (±5.1)
10a	>100	>100
10b	>100	>100
13e	50 (±6.5)	>100
14e	50 (±8.3)	>100
15a	20 (±7.0)	40 (±9.7)
15b	61 (±6.4)	85 (±6.6)
15c	20 (±4.2)	40 (±4.7)
15d	18 (±5.1)	38 (±3.8)
16a	42 (±8.1)	90 (±6.7)
16b	25 (±3.0)	50 (±5.9)
16c	10 (±3.0)	10 (±2.0)
16d	18 (±4.6)	38 (±4.6)
4	26 (±3.2)	47 (±5.6)
ATRA	11 (±3)	>100

[a]Results were obtained after 48 h of treatment. Cell cultures and evaluation of cytotoxicity and apoptosis were performed as previously described.[15]

Most of the retinoids tested in this study, including ATRA, 9-*cis*-RA and 13-*cis*-RA, were inactive (data not shown) in multidrug resistant HL60R cells, K562 leukemia and HUT78 T-cell lymphoma cells. HL60R cells overexpress P-glycoprotein (P-gp) and are resistant to apoptosis induced by drugs expelled out of cells by this multidrug transporter, such as daunorubicin, etoposide, vincristine, mitoxantrone and others. However, they are also resistant to apoptosis from drugs which are not substrates of P-gp, such as methotrexate, cytarabine, and cisplatin. K562 is a myeloid leukemia cell line expressing *BCR-ABL*, an oncogene that confers resistance to apoptosis from different cytotoxic drugs or radiations.

Moreover, K562 cells do not express Fas/APO1 a cell membrane receptor implicated in the activation of programmed cell death. In this study, we identified three new retinoids (**16b–d**) able to act in these cell lines with pleiotropic resistance. In HL60R, K562 and HUT78 cells, the compound **16c** showed a cytotoxic and apoptotic activity similar to that observed in the sensitive HL60 cell line. Also, **16b** was active in these cell lines, but at a concentration about 3–4 times higher than **16c**. On the contrary, **16d** was able to inhibit the growth, but not to induce programmed cell death (Table 2), in the resistant cells. These data indicate that **16c** may be considered an interesting drug for the therapy of different types of leukemia, especially those resistant to the conventional treatments and expressing factors like P-gp or the *BCR-ABL* oncogene.

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Table 2. Cytotoxic and apoptotic activity of retinoid derivatives **16b–d** in multidrug resistant HL60R cells and in 9-*cis*-retinoic acid resistant K562 and HUT78 cells[a]

	HL60R		K562		HUT78	
Compound No	IC_{50}	AC_{50}	IC_{50}	AC_{50}	IC_{50}	AC_{50}
16b	48 (±9.0)	87 (±8.9)	42 (±5.8)	50 (±6.1	22 (±7.2)	46 (±7.8)
16c	15 (±4.6)	25 (±5.5)	13 (±3.9)	27 (±6.8)	5 (±1.4)	10 (±3.8)
16d	25 (±2.1)	>100	50 (±8.8)	>100	43 (±7.9)	>100

[a]IC_{50} and AC_{50} were expressed as μM. Results were obtained after 48 h of treatment. Cell cultures and evaluation of cytotoxicity and apoptosis were carried out as previously described.[15]

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