

Retinoic acid and analogs as potent inducers of differentiation and apoptosis. New promising chemopreventive and chemotherapeutic agents in oncology*

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Abstract: In this report we will describe the preparation and the biological activity of a novel class of heterocyclic arotinoids endowed with potent cytotoxic and apoptotic activity. Structure–activity relationship studies revealed that the different stereochemistry at the C9 double bond of retinoids seems associated with a different biological activity: potent apoptotic activity for the *cis*-isomers, whereas differentiating activity for the *trans* structures. An interesting modified Wittig procedure that allows easily to arotinoids will also be described. The substitution of the alkenyl portion with a more flexible oxymethyl or aminomethyl moiety gave compounds with poor activity, whereas isoxazole-bridged arotinoids allowed compounds active also on multidrug-resistant (MDR) leukemia cell lines.

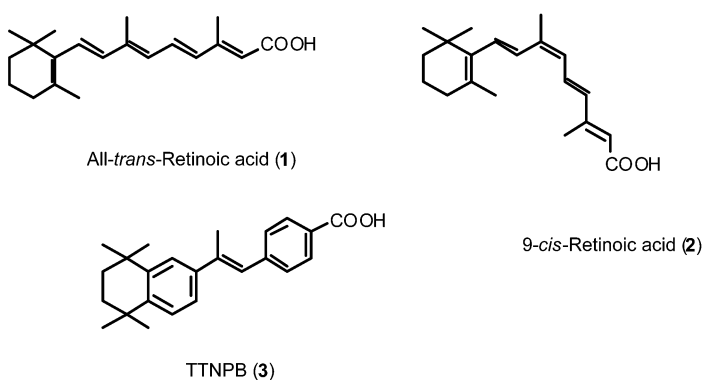
INTRODUCTION

Programmed cell death (PCD), 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 be involved. Recently, it has been shown that some natural and synthetic retinoids are able to induce apoptosis in cancer cell lines, and, also in this case, the mechanism is still unknown [4–7]. Retinoids are a class of natural and synthetic vitamin A analogs structurally related to all-*trans*-retinoic acid (ATRA) (**1**) [8,9]. Natural retinoids are also known to play a major role in regulating growth and differentiation of a wide variety of normal and malignant cell types, and, indeed, they can in various ways inhibit cell proliferation and induce differentiation and apoptosis, depending on the cell type. Retinoids

*Plenary lecture presented at the Hungarian–German–Italian–Polish Joint Meeting on Medicinal Chemistry, Budapest, Hungary, 2–6 September 2001. Other presentations are published in this issue, pp. 1387–1509.

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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 [8]. ATRA binds and activates the RAR receptors, while 9-*cis*-retinoic acid (9-*cis*-RA) (2) binds and activates both RAR and RXR receptors. 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 [10–12]. Encouraging preliminary clinical results have also demonstrated the importance of retinoids in combination chemotherapies [12]. 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 used to induce remission of acute promyelocytic leukemia (APL).

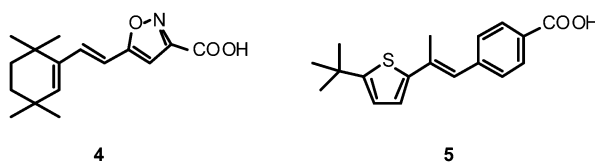


The ability of retinoids to regulate cellular processes *in vivo* is unfortunately associated with a high incidence of undesirable side effects. Consequently, a wider use of retinoids in dermatology (principally in the therapy of psoriasis and acne) and in other diseases such as in oncology (treatment of carcinomas and for cancer chemoprevention) has been precluded by unacceptable side effects including skin irritation, lipid and bone toxicity, visual effects, and teratogenicity.

In order to increase the selectivity toward the retinoid receptors and to obtain compounds of pharmacological interest, the relationships between structure and retinoid activity have been extensively studied by preparation of a large number of geometric isomers, as well as conformationally locked and/or restricted analogs, resulting in specific, rigid, three-dimensional configurations.

SHORT HETEROCYCLIC AROTINOIDS

At an early stage, we 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 heteroarotinoids and a class of new retinoid acid conjugates [13–16]. Among the isoxazole-heteroarotinoids, some compounds, such as 4 and 5, were found to be endowed with signifi-

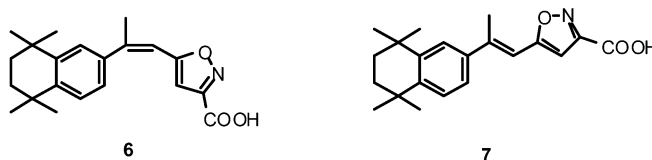


Synthetic Heteroretinoids

cant differentiating and antiproliferative activities; moreover, some new retinoic acid conjugates proved of interest for their antitumor activity and low toxicity [16].

More recently, we demonstrated that some new isoxazole-containing retinoids are endowed with interesting differentiating properties against human APL HL60 cells. We 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 **6** that is structurally related to arotinoids, was endowed with an interesting apoptotic activity. This derivative bearing the *cis* configuration at the double bond may resemble the 9-*cis*-RA but, unlike this natural compound, it does not bind the retinoid receptors. We found that the new compound was able to induce apoptosis in HL60 cells after only 24 h of treatment. The percentage of apoptotic cells in cultures treated with compound **6** was 23%, 60%, and 90% after 24, 48, and 72 h, respectively. This apoptosis-inducing activity was 6.5 and 4 times higher than that of 13-*cis*-retinoic acid and 9-*cis*-retinoic acid respectively, while ATRA at the concentration of 5×10^{-5} M was unable to induce apoptosis. Interestingly, the *trans* isomer **7** was only a poor inducer of apoptosis, but still retained an appreciable differentiating activity in HL60 cells.

Thus, we have considered **6** as a possible new lead for the development of novel apoptosis-inducing agents structurally related to retinoids and targeted to the cell cycle.

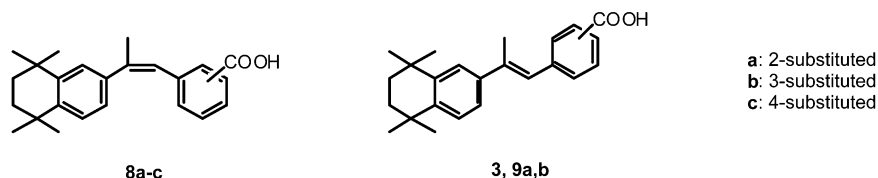


PROGRAMMED CELL DEATH ASSOCIATED WITH THE STILBENE MOTIF OF AROTINOIDS

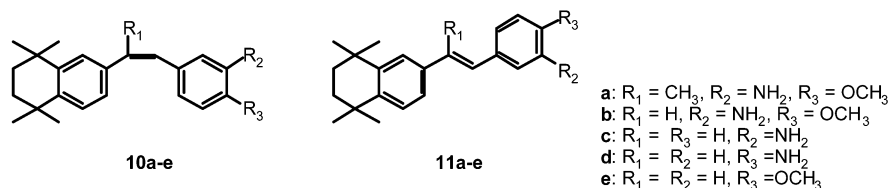
Since apoptosis is probably the major modality by which leukemic cells are killed by treatment with pharmacological agents, it is likely that the identification of new retinoids able to induce apoptosis in different types of tumor cells, independently/or not of their differentiating activity, could be an important goal in the cancer chemotherapy. Therefore, we focused our attention on the identification of the structural features associated with the apoptotic activity of compounds related to arotinoids. Considering that the *E* isomer **7** was found to be endowed with an appreciable differentiating activity while the *cis*-stilbene derivative **6** was essentially inactive at level of the retinoid receptors, but was identified as a potent inducer of apoptosis, we hypothesized the existence of a non-RAR-mediated mechanism associated with the *cis*-stilbene-like motif of **6** and responsible for the apoptotic activity of the compound. On the other hand, although 4-HPR and CD437 activate nuclear receptors, their proapoptotic activity appears to be mediated, in part, through retinoid receptor-independent pathways.

In addition, 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 olefine contained in **6**, may be related to the apoptotic activity of these compounds. Consequently, we planned the synthesis of TTNPB-retinoid analogs **8a-c** and **9a,b**, taking into consideration both the configuration *E* and *Z* of the stilbene motif [18]. Thus, we discovered that the *cis*-TTNPB **8c**, which still retains some structural features of 9-*cis*-RA, is endowed with a potent apoptotic activity, being more active than the previously described isoxazole retinoid **6**. On the other hand, TTNPB **3**, a known potent differentiating arotinoid, is only a poor inducer of apoptosis. We discovered also that the amino **10c** proved to be a particularly potent apoptosis-inducing agent active in MDR cell lines, in particular resistant to the apoptotic effects of several chemotherapeutic drugs, such as daunorubicin, methotrexate, citarabine, 5-fluorouracil, and others. In particular, in HL60R, K562, and HUT78 cells, the compound

10c showed a cytotoxic and apoptotic activity similar to that observed in the sensitive HL60 cell line. Also, **10b** was active in these cell lines, but at concentration about 3–4 times higher than **10c**. On the contrary, **10d** was able to inhibit the growth, but not to induce PCD in the resistant cells. These data indicate that **10c** 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.



In summary, we observed previously that isoxazole arotinoids with the *cis* stereochemistry were more efficient inducers of apoptosis than their correspondent *trans* stereoisomers. We observed also 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* compound **11a**, which is twice more active in inducing PCD than the respective *cis* isomer **10a**. However, in the case of the most active compound **10c**, the *cis* isomer is clearly the best apoptosis-inducing agent.



Therefore, although we hypothesized that the different stereochemistry of the double bond may be associated with a different apoptotic activity, this cannot 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 to confer cytotoxic and apoptotic activity to this class of retinoids. Thus, we consider that 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 new retinoids will also be important in studies of receptor selectivity aimed to better clarify the mechanism of action of vitamin A and of its analogs (retinoids).

STRONG BICYCLIC GUANIDINE BASE-PROMOTED WITTIG REACTION: AN EASY SYNTHETIC APPROACH TO AROTINOIDS

In our continued search for reactions in which strong guanidine bases may provide improved synthetic methodologies over conventional approaches [19,20], we have found that 1,5,7-triazabicyclo[4,4,0]dec-5-ene (**12**) and its 7-methyl analog (MTBD, **13**) may represent, in some cases, surprisingly use-

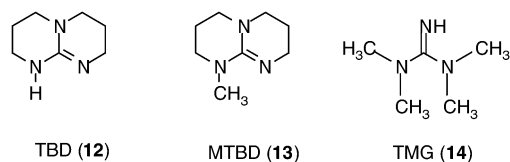


Table 1 Cytotoxic and apoptotic activity of different retinoid derivatives in HL60 cells.

Compound No.	IC ₅₀ (μM)	AC ₅₀ (μM)*
3	46 (±6.2)	>100
8a	52 (±7.3)	90 (±7.8)
8b	50 (±10)	80 (±9.8)
8c	10 (±2.8)	40 (±5.1)
9a	>100	>100
9b	>100	>100
10a	42 (±8.1)	90 (±6.7)
10b	25 (±3.0)	50 (±5.9)
10c	10 (±3.0)	10 (±2.0)
10d	18 (±4.6)	38 (±4.6)
10e	50 (±8.3)	>100
11a	20 (±7.0)	40 (±9.7)
11b	61 (±6.4)	85 (± 6.6)
11c	20 (±4.2)	40 (±4.7)
11d	18 (±5.1)	38 (±3.8)
11e	50 (±6.5)	>100
6	26 (±3.2)	47 (±5.6)
ATRA	11 (±3)	>100

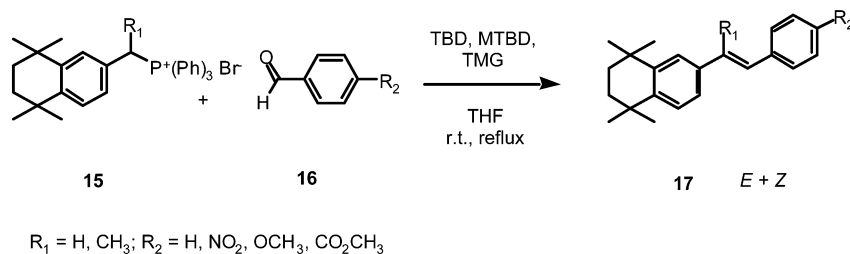
*Results were obtained after 48 h of treatment.

ful new promoters of the Wittig reaction. When TBD and MTBD were applied to affect the Wittig reaction, unexpected results were obtained in terms of high yield and mildness of reaction conditions.

Our procedure is simpler than that using organolithium compounds or other ionic strong bases, and has the added advantage that it can be used to prepare phosphoranes from compounds containing functional groups, such as the carboxylic ester groups, which would react with organolithium compounds or during the work-up in the presence of a strong basic medium. The scope of the methodology was explored using a representative panel of aldehydes and phosphonium salts; reactions were attempted in many cases with TBD, MTBD, or TMG (**14**). The most promising promoter appeared to be the bicyclic guanidine base TBD; with this base yields were in many cases good or very good [21].

It is remarkable that by means of the proposed methodology unstable ylids may be generated in high yields. Indeed, the alkyltriphenylphosphonium salts reacted efficiently in the presence of TBD with aromatic aldehydes.

As we needed access to the class of stilbene analogs described above, our attention was drawn by the idea that the strong bicyclic guanidine bases TBD or MTBD could be effective promoters of the reaction between the phosphonium salts **15** and the *p*-methoxycarbonylbenzaldehyde **16** (Scheme 1).

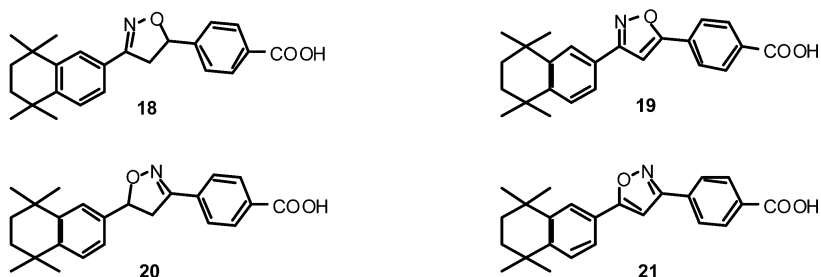
**Scheme 1**

Thus, while during the years this reaction has been largely described by means of strong ionic bases in an inert atmosphere, we found that with TBD the corresponding stilbene derivatives may be obtained in appreciable yields, comparable with, and in many cases superior to, those described in the literature. In particular, TTNPB was obtained in 55% yield, whereas its demethyl analog **17** ($R_1 = H$, $R_2 = CO_2Me$) in 90% yield. Interestingly, compound **17** was also obtained in 83% yield in absence of anhydrous conditions.

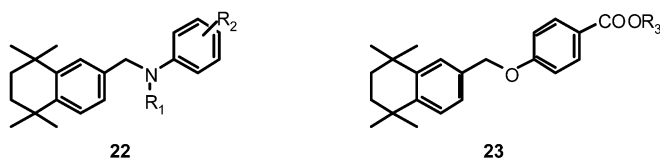
In summary, although the literature enumerates hundreds of different ionic base-promoted Wittig procedures, the simplicity, environmental friendliness, and low costs make our procedure, at least in some cases, a practical alternative. The additional advantages of operation and work-up simplicity make this methodology a serious candidate for widespread industrial applications such as generating combinatorial stilbene libraries.

NOVEL STERICALLY RESTRICTED AND MORE FLEXIBLE AROTINOIDS [22]

Because the stereochemistry of the C9 alkenyl portion of natural 9-*cis*-RA, which corresponds to the only olefinic moiety in our lead **6**, seemed of particular importance for apoptotic activity, we planned the synthesis of new retinoid analogs using a sterically restricted flexibility in this region. Thus, the alkenyl basic motif of TTNPB was replaced by an isoxazole (compounds **19** and **21**) or an isoxazoline moiety (compounds **18** and **20**) which may stiffen the molecule; *vice versa*, a flexible connecting amino- or oxymethyl moiety, which may enable the system to better “fit” the receptor, was introduced in derivatives **22** and **23**.



Since 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, 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. Our designed heterocyclic retinoids **18–21**, however, still retain some structural features of the lead compound **6**, i.e., the isoxazole heterocycle and tetrahydrotetramethylnaphthalenyl ring. On the other hand, derivatives **22** and **23** also contain a heteroatom in their structure. We therefore speculated that such new retinoid derivatives might be still endowed with apoptotic activity. All the new retinoids were tested for their differentiating, cytotoxic, and apoptotic activities. In addition, the ability of the compounds to regulate the retinoid receptors *in vitro* was evaluated using transcriptional activation assays.



We found that the cytotoxic and apoptotic effects of **21** on HL60 cells were markedly greater than that of **18**, **19**, **20** and **22**, **23**. The compound was able to induce apoptosis at concentrations lower than 10 μ M. In this respect, it was also more active than the classical retinoids ATRA, 13-*cis*-RA, and 9-*cis*-RA, and than the previously described isoxazole retinoid **6**.

Compounds **18–21** 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 retinoid receptors in their natural state was evaluated in a vulvar carcinoma cell line, SW962, which expresses all of the known human RAR and RXR receptors. Compound **21** induced the highest level of activity, which was 97% of the activity level induced by 9-*cis*-RA.

In summary, the isoxazole arotinoid **21** represents a novel retinoid endowed with potent apoptotic activity in MDR cells. The ability of **21** to act in K562 and HL60R cell lines suggests that this compound may have important implications in the treatment of different leukemias. The ability of **21** to act in this cell line in a manner similar to the other *BCR-ABL* negative cell lines implies that it may be an effective treatment for chronic myelogenous leukemia and acute lymphoblastic (ALL), as well as non-lymphoblastic (ANLL) leukemias expressing the *BCR-ABL* oncogene. Also, the ability to induce apoptosis in HL60R cells represents an important property for a possible clinical use of **21**. We have previously observed that HL60R is resistant to drug-induced apoptosis independently of its expression of the P-glycoprotein. This resistance seems to be correlated to the presence of high constitutively activated levels of the transcription factor NF- κ B, which is not observed in the parental sensitive HL60 cells. Thus, **21** may be a drug useful both in MDR and in apoptosis-resistant malignancies.

CONCLUSIONS

As a result of our quest for new retinoid-related compounds capable of activating selectively mechanisms for apoptosis in cancer cells but devoid of undesirable side effects, we found some structural features that seem to be important to confer apoptosis vs. differentiation activity to well-known and newly built molecules. The steric conformational block in the *cis* or *trans* double bond between the aromatic components has been located as a major, even though not absolute, discriminating factor to address the mechanism of action. A substitution of the alkenyl moiety with an isoxazole ring led us to discover a potent inducer of apoptosis active also on multidrug-resistant cell lines. On the contrary, an introduction of a more flexible oxymethyl or aminomethyl instead of the double bond gave only inactive products. Moreover, an alternative, practical Wittig reaction procedure has been developed during our synthesis of stilbene-like compounds.

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