Communications to the Editor

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DIFFERENTIATION INDUCERS OF HUMAN PROMYELOCYTIC LEUKEMIA CELLS HL-60. AZOBENZENECARBOXYLIC ACIDS AND STILBENECARBOXYLIC ACIDS

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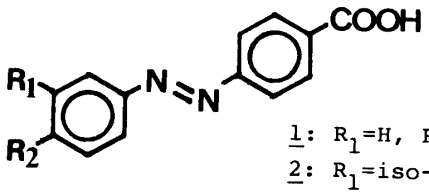
Azobenzene-4-carboxylic acid derivatives are a new type of differentiation inducers of human myelogenous leukemia cell line HL-60. Induced cells are morphologically and functionally mature granulocytes. A study of substituent effects indicated that these compounds are related to the known synthetic retinoid, tetrahydrotetramethylnaphthylpropenylbenzoic acid.

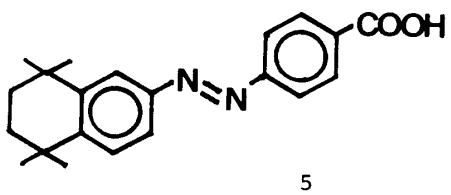
KEYWORDS—— HL-60; differentiation; azobenzene-4-carboxylic acid; stilbene-4-carboxylic acid; retinoic acid

The human myelogenous leukemia cell line HL-60 was established by Gallo[1] from a patient with acute promyelocytic leukemia. The cell line is known to be induced to differentiate terminally into mature macrophages and granulocytes *in vitro* by various compounds such as DMSO,[2] vitamin D_3,[3] retinoic acid,[4] etc. Induction of terminal cell differentiation of such cells has potential applicability to the chemotherapy of human leukemia.[5] However, the known inducers are nonspecific or too toxic. Thus, we have been searching for new types of differentiation inducers of HL-60 cells. Very recently, we reported two kinds of inducers, a terephthalic anilide derivative (Am80)[6] and a chalcone-4'-carboxylic acid derivative (Ch55).[7] In this paper we report on the differentiation-inducing activities of alkyl-substituted azobenzene-4-carboxylic acids and their relationship to stilbene-4-carboxylic acid derivatives.

The HL-60 cell line has been maintained in continuous suspension culture. Cells are cultured in plastic flasks in RPMI-1640 medium supplemented with 5% fetal calf serum and antibiotics (penicillin G and streptomycin). Cells were cultured with test compounds for 4 days, and stained with Wright-Giemsa. Differential counts were then performed on a minimum of 200 cells under a light microscope. Nitroblue



1: R_1=H, R_2=H
2: R_1=iso-Pr, R_2=H
3: R_1=t-Bu, R_2=H
4: R_1=iso-Pr, R_2=iso-Pr



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tetrazolium (NBT) reduction was assayed as described.[8] Cells were incubated for 20 min at 37°C in RPMI-1640 medium (5% FCS) and an equal volume of phosphate-buffered saline containing NBT (0.2%) and 12-O-tetradodecanoylphorbol-13-acetate (200 ng/ml). The percentage of cells containing blue-black formazan was determined.

Azobenzene-4-carboxylic acid derivatives were prepared from alkyl-substituted aniline and methyl p-nitrosobenzoate in acetic acid, followed by mild alkaline hydrolysis under argon gas.

Nonsubstituted azobenzene-4-carboxylic acid (1) was inactive at 10^{-6} M or below. As in the case of terephthalic anilide derivatives, introduction of a bulky alkyl group such as an isopropyl or tert-butyl group at the meta position resulted in differentiation-inducing activity (compound 2, 3). These compounds induced HL-60 cells to differentiate into mature granulocytes, which were detected on the basis of cell morphology and functional ability to reduce NBT. Dose-response curves for NBT reduction assay are shown in Figure 1. The ED_{50} values of the inducers were calculated from the NBT assay data, which parallel the morphological change. Derivatives having only the para-alkyl group did not exhibit any activity (data not shown), but 4, which has two alkyl groups at the meta and para positions, was more active than 2. The ED_{50} of 4 is 2×10^{-9} M similar to that of retinoic acid (the positive control in our assay). Furthermore, compound 5 which has the two alkyl groups fixed by a ring system, was several times more active than retinoic acid (ED_{50} of 5, 5×10^{-10} M).

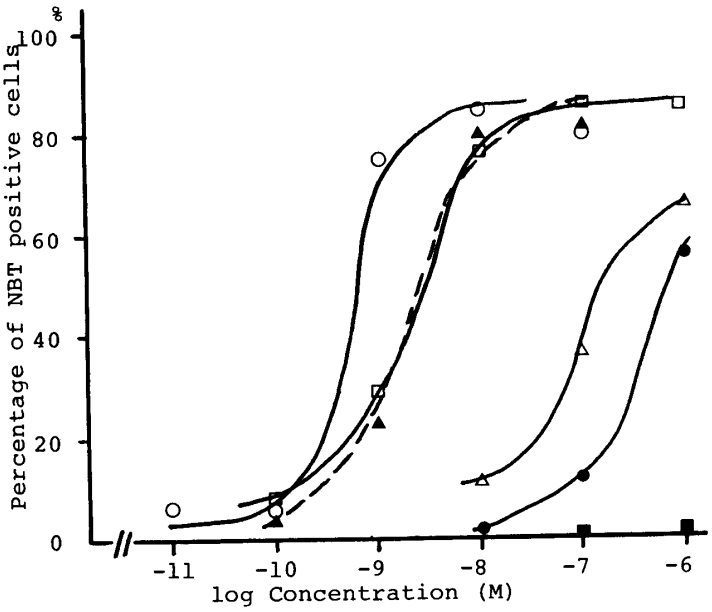
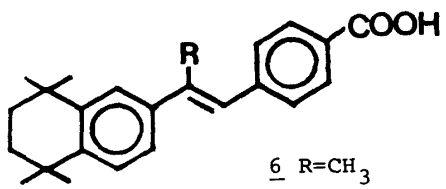


Figure 1. Dose-Response Curves for Azobenzene-4-carboxylic Acids in the Nitroblue Tetrazolium Reduction Assay

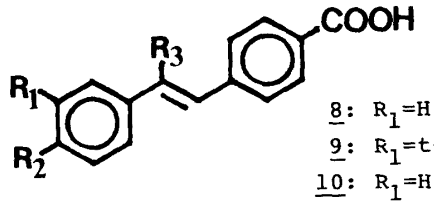
▲ , retinoic acid
■ , 1
● , 2
△ , 3
□ , 4
○ , 5

A stilbene derivative, tetrahydrotetramethylnaphthylpropenylbenzoic acid (TTNPB, 6) has been reported to inhibit chemical carcinogenesis[9] and to induce differentiation of HL-60 cells.[10] Azobenzene and stilbene are isoelectronic. From the results with the azobenzene derivatives, it appears that the cyclohexenyl ring system of TTNPB may not be essential for the activity. Therefore, the activities of several alkyl-substituted stilbene-4-carboxylic acids were examined. They were prepared from an acetophenone or benzaldehyde derivative and phosphonium salt by means of the Wittig reaction, followed by hydrolysis.



6 R=CH_3

7 R=H



8: R_1=H, R_2=H, R_3=H
9: R_1=t-Bu, R_2=H, R_3=H
10: R_1=H, R_2=t-Bu, R_3=H
11: R_1=iso-Pr, R_2=iso-Pr, R_3=CH_3

TTNPB 6 also induced HL-60 cells to develop into mature granulocytes in our system. The ED_{50} of 6 was 5 x 10^{-10} M which is the same as that of 5. Since compound 7 was as active as 6, clearly the methyl group on the ethylene of TTNPB is not necessary for the activity. Dose-response curves for the stilbene series are shown in Figure 2. In this case again, the simple stilbene-4-carboxylic acid 8 was completely inactive, and compounds having an alkyl group at the meta position such as 9, but not at the para position such as 10, possessed the inducing activity. Compound 11 corresponding to the azobenzene derivative 4 was more active, and its ED_{50} is 6 x 10^{-9} M, which is comparable to the ED_{50} of retinoic acid, 2 x 10^{-9} M. These results show that the cyclohexenyl ring system of TTNPB is not essential for the inducing activity. The moiety seems to act only as a bulky hydrophobic group.

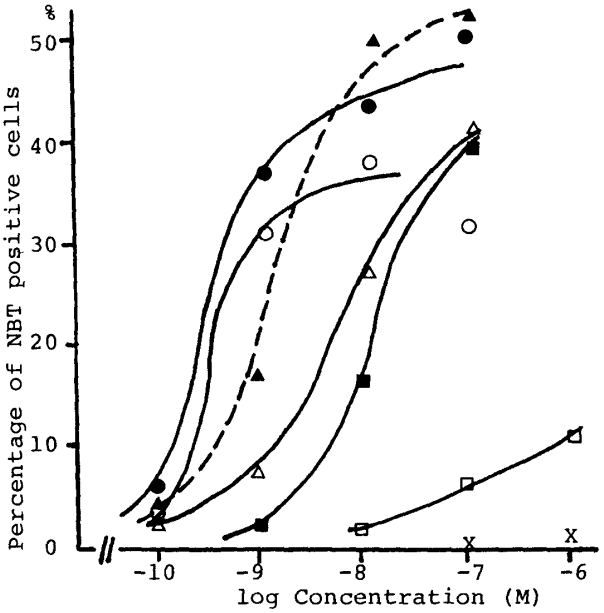


Figure 2. Dose-Response Curves for Stilbene-4-carboxylic Acids in the Nitroblue Tetrazolium Reduction Assay

▲ , retinoic acid
○ , 6
● , 7
X , 8
■ , 9
□ , 10
△ , 11

The two kinds of inducers, azobenzene-4-carboxylic acids and stilbene-4-carboxylic acids, both cause HL-60 cells to differentiate into mature granulocytes, as retinoic acid, Am80 and Ch55 do, and the alkyl substituent effects of all of them are very similar. Firstly, the bulky meta-alkyl group is very important for the activity. Secondly, the para-alkyl group itself causes no activity, but when it coexists with the meta-alkyl group, it enhances the activity. Thirdly, the compounds having a tetrahydrotetramethylnaphthalene ring, which is regarded as "fixing" the alkyl groups, are more active than retinoic acid. Because of these similarities among azobenzene-4-carboxylic acid derivatives and stilbene-4-carboxylic acid

derivatives, they are considered to be agonists. Moreover, our synthetic compounds previously reported, terephthalic anilides and chalcone-4'-carboxylic acids, also show similar properties, and may be agonists.

TTNPB was designed as a retinoic acid derivative and seems to be structually related to retinoic acid (X-ray crystallography).[9] One of the two dimethyl groups on the tetraline ring of the new compounds, that is, the important meta-alkyl group, can be regarded as corresponding to the dimethyl group on the cyclohexenyl ring of retinoic acid. Thus, the structure-activity relationship suggests that these inducers can be classified as retinoids.

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