## Communications to the Editor

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# NEW TYPE INDUCERS OF DIFFERENTIATION OF HUMAN HL-60 PROMYELOCYTIC LEUKEMIA CELLS. TEREPHTHALIC ANILIDES

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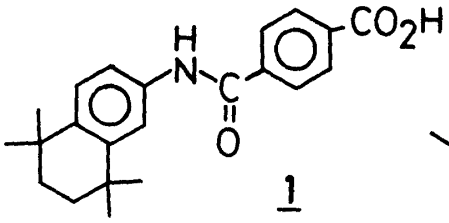
Induction of terminal differentiation of human myelogenous leukemia cells, HL-60, was caused by terephthalic acid mono-5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthylamide. The differentiated cells are morphologically and functionally granulocyte-like. The minimum concentration for detectable activity is less than $10^{-10}$ M (0.04 ng/ml), and differentiation reaches a maximum at about $10^{-8}$ - $10^{-9}$ M.

KEYWORDS —— cell differentiation; HL-60; leukemia; terephthalic anilide; promyelocyte; retinoic acid

Induction of differentiation of human acute myelogenous leukemia cells may have implications in the therapy of the human leukemia and of other malignant and premalignant disorders.[1] However, only a couple of specific inducers have been found. The most important compounds are retinoic acid and its related compounds (retinoids),[2,3] which can induce granulocyte differentiation of human HL-60 cells.[4] More recently, 1,25-$(OH)_2$-$D_3$ has been reported as an inducer of the same cells.[5] In relation to our studies on epigenetic modulators of cell growth, several synthetic compounds, which were designed from structure-activity consideration of retinoids and of teleocidins and indolactams,[6] were tested for their inducing activity of differentiation of HL-60 cells. Some terephthalic anilides are found to exhibit the activity at very low concentrations.

The HL-60 cell line (established by Gallo)[7] was supplied by Prof. Takaku and has been maintained in continuous suspension culture. Cells are cultured in

plastic flasks in RPMI-1640 medium supplemented with 5% heat inactivated fetal calf serum and antibiotics (penicillin G and streptomycin). Cells were cultured with inducers for 4 days, and stained with Wright-Giemsa. Differential counts were then performed under light microscopy on a minimum of 200 cells. Nitroblue 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 buffer saline containing NBT (0.2%) and 12-O-tetradodecanoylphorbol-13-acetate (200 ng/ml). The percentage of cells containing blue-black formazan was determined. Terephthalic anilides were prepared from methyl terephthaloyl chloride and the corresponding anilines: terephthalic acid mono-5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthylamide, 1, mp 205.5-206°C; 2 ($CH_3$ ester of 1), mp 211-212°C; methyl terephthalic mono-3,4-diethylanilide, 3, mp 122-123°C; 4 (N-methyl derivative of 2), mp 117-118°C.

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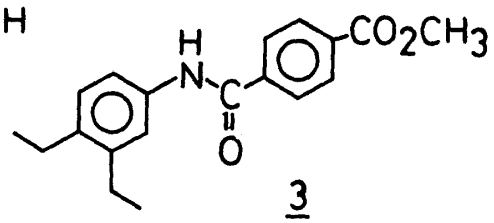


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In the absence of any inducer, HL-60 cells are predominantly typical promyelocytes with large round nuclei, each containing nucleoli and dispersed nuclear chromatin. The addition of $10^{-9}$ M 1 or 2 to the culture induced in a majority of the cells a striking morphological change characteristic of terminal differentiation of myeloid cells. The morphological changes induced by 1 are illustrated in Fig. 1. The induced cell exhibits the following changes: smaller nuclear size, decreased nuclear/cytoplasmic ratio, less prominant cytoplasmic granules, marked reduction or complete dissapearance of nucleoli. Some showed marked indentation

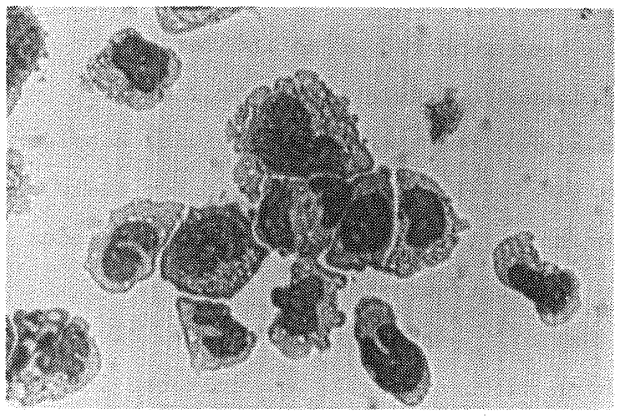

Fig. 1. Morphology of Induced HL-60 Cells Cultured in $10^{-9}$ M 1 for 4 Days

Cytospin slide preparations of suspension cell cultures stained with Wright-Giemsa (x400). Cells in this Fig. consist of metamyelocytes and banded neutrophils.

and segmentation of the nuclei (banded and segmented neutrophils). The extent of differentiation at various concentrations of the drugs indicates that 1 and 2 have a higher activity than retinoic acid (Table 1).

Table 1. Differential Counts of HL-60 after Incubation with Inducing Compounds

| Inducer | Conc. (M) | Myeloid cell type, % of total cells | | |
|---|---|---|---|---|
| | | Promyelocytes | Myelocytes and metamyelocytes | Banded and segmented neutrophils |
| None | | 96 | 4 | 0 |
| Retinoic acid | $10^{-9}$ | 15 | 70 | 15 |
| | $10^{-10}$ | 88 | 11 | 1 |
| 1 | $10^{-9}$ | 13 | 75 | 12 |
| | $10^{-10}$ | 26 | 61 | 13 |
| | $10^{-11}$ | 95 | 5 | 0 |
| 2 | $10^{-9}$ | 12 | 75 | 13 |
| | $10^{-10}$ | 57 | 38 | 5 |
| | $10^{-11}$ | 93 | 7 | 0 |
| 3 | $10^{-7}$ | 75 | 23 | 2 |
| 4 | $10^{-7}$ | 96 | 4 | 0 |

The diethyl derivative (3) is slightly, but definitely, active. Functional changes in HL-60 cells induced by 1, 2, and 3 were tested by NBT reduction which provides a sensitive differentiation marker associated with morphological change (Fig. 2). After four days of incubation in the presence of various concentrations of 1, the percentage of HL-60 cells reducing NBT varied with concentration, reaching a maximum at $10^{-9}$ M in accordance with the morphological changes.

With the addition of the inducer, alterations in the growth rate of the cells were apparant: growth ceased by day 4, in accordance with the inability of the matured differentiated cells to proliferate. The high percentage of viable cells eliminated the possibility that all or a major part of the induced differentiation was a result of selective enrichment for differentiated cells.

The effect of an alkyl substituent on the aniline ring seems to be important, since the unsubstituted and simple alkyl substituted compounds (data not shown) are not active, or far less active, than 1 and its methyl ester 2. An alkyl group on the nitrogen atom (i.e., 4) seems to abolish the inducing activity. A systematic study of structure-activity relationship is in progress.

It is surprising that the activity of 1 and 2 are so high. As their chemical

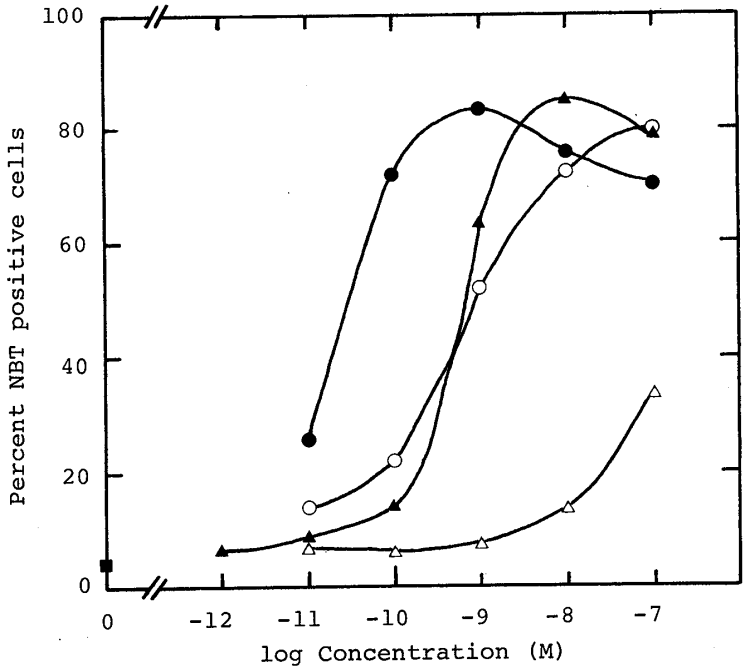


Fig. 2. Dose-Response Curves for Terephthalic Anilides in the Nitroblue Tetrazolium Reduction Assay

▲, retinoic acid;
●, 1;
○, 2;
△, 3;
■, blank.

structure is very different from retinoic acid, other novel biological functions of 1 and 2, and their inducing activity on other human cell lines on which retinoic acid has no effect will be urgently explored. The discovery of this new inducer will have great impact on the study of differentiation of blood cells and the chemotherapy of cancer.

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