Biochemical Pharmacology, Vol. 34, No. 12, pp. 2077–2082, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00
Pergamon Press Ltd.

INDUCTION OF HAEMOGLOBIN SYNTHESIS IN THE HUMAN LEUKAEMIA CELL LINE K562 BY MONOMETHYLTRIAZENES AND IMIDAZOTETRAZINONES

MICHAEL J. TISDALE

Cancer Research Campaign Experimental Chemotherapy Group, Department of Pharmacy, University of Aston in Birmingham, Birmingham B4 7ET, U.K.

(*Received* 8 *May* 1984; *accepted* 20 *December* 1984)

Abstract—The effect of a group of imidazotetrazinones and alkyltriazenes on the growth and the induction of differentiation associated properties in K562 human erythroleukaemia cells has been investigated. Only 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one and a monomethyltriazene induced the formation of benzidine-positive cells, and a linear relationship was observed between growth inhibition and the percentage of haemoglobin producing cells in the culture. However, growth inhibition alone was insufficient for induction of haemoglobin synthesis since other members of the series inhibited growth without substantially increasing the number of haemoglobin-producing cells. Induction was accompanied by an increase in cell size, and appeared not to arise by selective toxicity to the original non-differentiated cell compartment followed by clonal expansion of the differentiated cells. These results suggest that methylating agents are more effective than ethylating agents in the alteration of gene expression.

3,3-Dimethyltriazenes are broad spectrum antitumour agents [1–3] which are thought to be activated by metabolic oxidation followed by *N*-demethylation [2]. Structure–activity relationships suggest that at least one methyl group must be present for antitumour activity, and the diethyltriazenes are inactive antitumour agents, although *N*-ethyltriazenes are capable of alkylating DNA [4].

Our group has been studying the structure–activity relationship of a group of imidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3*H*)-ones, some of which have potent antitumour activity [5]. These compounds are capable chemically of generating cytotoxic species similar to those produced by both the antitumour nitrosoureas and triazenes. Thus 3-(2-chloroethyl) and 3-methyl substitution of the ring yields highly active agents, while 3-ethyl substitution results in inactivity against the TLX5 mouse lymphoma [6].

Alkyl derivatives of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), methyl nitrosourea (MNU), methyl methanesulphonate and dimethyl sulphate have been shown to induce irreversible differentiation of mouse neuroblastoma cells in tissue culture as judged by neurite formation and an increase in the activity of acetylcholinesterase [7,8]. The frequency of the induction of neurite formation by the methyl derivatives was higher than that caused by their corresponding ethyl derivatives, although higher concentrations of the latter were used to induce cell growth inhibition to the same extent as their methyl derivatives [8].

Several models for *in vitro* differentiation now exist. We have chosen the K562 cell line to investigate gene expression induced by monomethyltriazenes and imidazotetrazinones since haemoglobin synthesis can be induced by various low molecular weight compounds [9]. This line was originally established from the pleural effusion of a patient with chronic myelogenous leukaemia in terminal blast crisis [10] and has been considered to represent an early stage of differentiation of the granulocyte lineage [11]. Surface markers for both myeloid and megakaryocytic lineages are expressed in addition to erythroid [12].

MATERIALS AND METHODS

Tissue culture medium RPMI 1640, foetal calf serum and newborn calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). The triazenes and imidazotetrazinones were synthesized by Professor M. F. G. Stevens and Dr. M. D. Threadgill in this department and by May and Baker Ltd. (Dagenham, Essex).

Cell culture conditions and determination of differentiation. Cells were maintained at 37° in RPMI 1640 media containing 10% calf serum (either foetal or newborn as stated in Results) under an atmosphere of 10% CO_2 in air. Drug solutions were made up in dimethyl sulphoxide (DMSO) at 10-times the required concentration such that the final concentration of DMSO in the culture medium did not exceed 0.5%. At this concentration DMSO does not induce erythroid differentiation in K562 cells [9]. For induction experiments cells were plated at a concentration of 3×10^4/ml in the wells of a multiwell dish (Nunc) and cell number was determined daily with a Coulter counter model D. The cells were sedimented by centrifugation (300 *g*, 10 min) prior

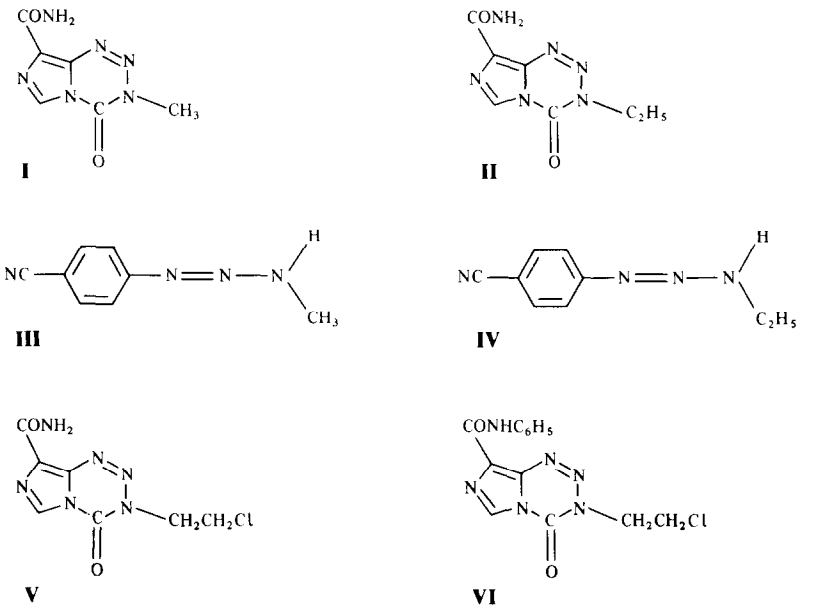

Fig. 1. Structure of chemicals used in the study.

to measurement of erythroid differentiation which was scored by benzidine staining. 3,3,5,5′-Tetramethylbenzidine (2 mg/ml in 1% acetic acid) was mixed with 30% hydrogen peroxide 20 μl/ml) and added directly to an equal volume (10 μl) of cell suspension. After 5 min cells were scored as benzidine positive (blue) using an Olympus phase contrast microscope at 40×. Viability was determined by trypan blue exclusion.

RESULTS

The chemical structures of the agents used in this study are shown in Fig. 1. All of the agents inhibit growth at the concentrations used in this investigation. The effects of 8-carbamoyl-3-methylimidazo [5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (I) on the replication of K562 cells is shown in Fig. 2. The initial rates of cell growth are affected by concentrations of I greater than 1 μg/ml and at 15 μg/ml growth is almost completely suppressed. However, trypan blue exclusion is not reduced during the first 3 days of culture and by day 4 the percentage of dye excluding cells in cultures treated with high concentrations of I is actually increased over the untreated control (Table 1), although fewer cells are present.

The inhibition of cell growth produced by I is accompanied by an increase in benzidine-positive cells during culture. The frequency of production of haemoglobin producing cells increases with concentration up to a maximum of 15 μg/ml and with time of incubation (Fig. 3), the first increase in benzidine positive cells being apparent 3 days after drug exposure. Both I and the methyltriazene (III) produce an exponential increase in the number of benzidine-positive cells above a concentration of 5 μg/ml (Fig. 4), although lower concentrations of the

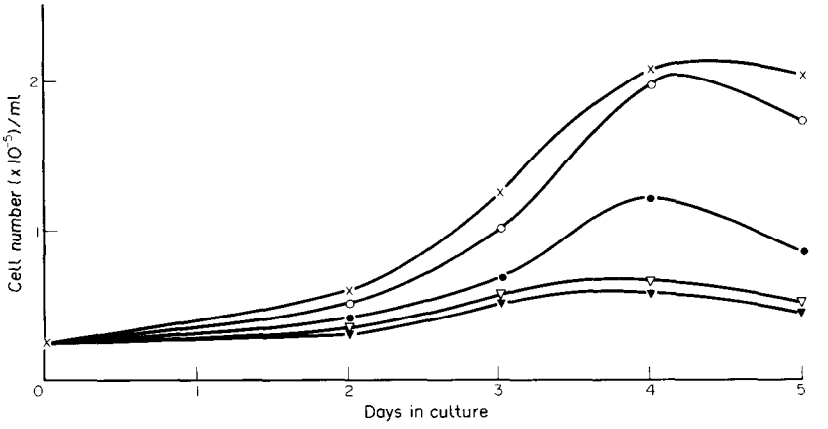

Fig. 2. Growth inhibition of K562 cells cultured in the absence (×) or in the presence of 1(▽), 5(●), 7.5(○) or 15(▼) μg/ml of I. The experiment was repeated six times and is a representative result.

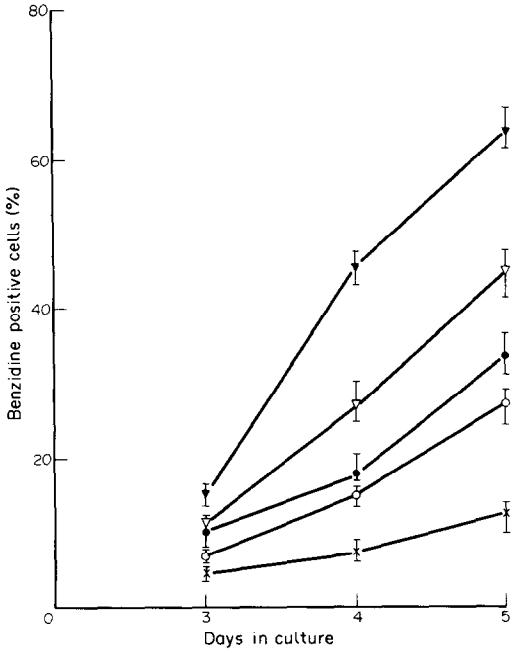

Fig. 3. Kinetics of appearance of benzidine-positive K562 cells cultured in the absence (×) or in the presence of 1(○), 5(●), 7.5(▽) or 15(▼) μg/ml of I. The percent benzidine-positive cells was evaluated on a minimum of 200 cells counted in a hemocytometer and was repeated 4 times.

drugs also cause a significant increase in haemoglobin production. In contrast to the methyl derivatives the corresponding ethyl analogues (II and IV) are ineffective in the induction of benzidine-positive cells, even at concentrations which have an equivalent effect on cell growth (Fig. 5 and Fig. 6).

Although the methyltetrazinone (I) and the methyltriazene (III) are more growth inhibitory than the ethyl derivatives (II and IV) (ID_{50} 3.0 vs 28 μg/ml and 0.9 vs 32 μg/ml respectively) growth inhibition alone is insufficient for induction of haemoglobin syntheses in K562 human erythroleukaemia cells. For the methyl analogues there is a linear relationship between growth inhibition and the induction of benzidine-positive cells (Figs. 5 and 6) while for

Table 1. Exclusion of trypan blue by K562 cells 4 days after treatment with I

Concentration (μg/ml)	Percentage of dye excluding (cells) (±S.E.M.)*
0	75 ± 5
0.5	54 ± 8
1	60 ± 6
2.5	71 ± 9
7.5	79 ± 8
10	85 ± 5
15	95 ± 2

* Results are means of three experiments and counts were made on 200 cells for each drug concentrations.

the ethyl analogues there is no correlation between growth inhibition and the induction of erythroid differentiation. Cytosine arabinoside a known inducer of haemoglobin synthesis in K562 cells [9] also shows a linear relationship between growth inhibition and the induction of benzidine-positive cells (Fig. 5). Other imidazotetrazinones and the alkylating agent chlorambucil also show no correlation between growth inhibition of K562 cells and the induction of benzidine-positive cells (Fig. 7). The extent of induction of haemoglobin synthesis with these agents is variable and in most cases is not statistically significant. Thus out of the compounds listed in Fig. 1 only the methyltetrazinone (I) and the methyltriazene (III) are effective in the induction of haemoglobin synthesis of K562 cells, although all of the agents inhibit cell growth.

The induction of benzidine-positive cells by I does not appear to arise from a selective toxicity towards the undifferentiated cell compartment, thus permitting the differentiated cells to proliferate. After treatment with 15 μg/ml of I by day four 46 ± 5% of the cell population stain for haemoglobin, whereas a maximum of 29% could have arisen by clonal expansion of the differentiated cell population. Similarly at 1 μg/ml of I the corresponding figures are 15 ± 3% and 9%.

Also suggesting a specific inductive effect by I, which is independent of its growth inhibitory effect,

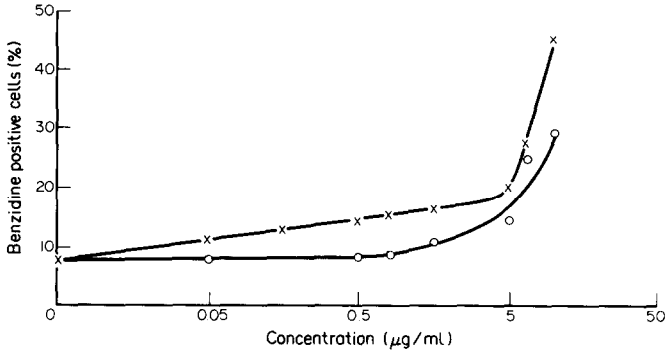

Fig. 4. Dose–response relationship of the induction of benzidine-positive K562 cells by I(×) and III(○) after 4 days in culture. The curves are averages of 4 determinations which did not differ by more than 10%.

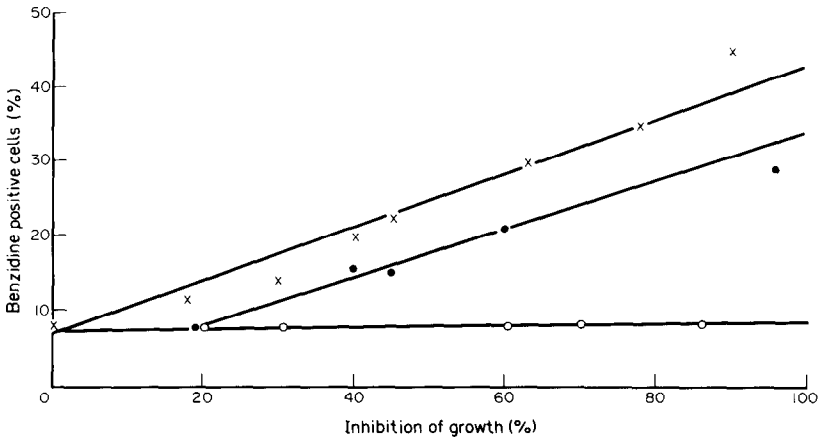

Fig. 5. Relationship between growth inhibition of K562 cells and induction of benzidine-positive cells by I(×), II(○) and Ara C(●), after 4 days in culture. The results are an average of 4 determinations which did not differ by more than 10%.

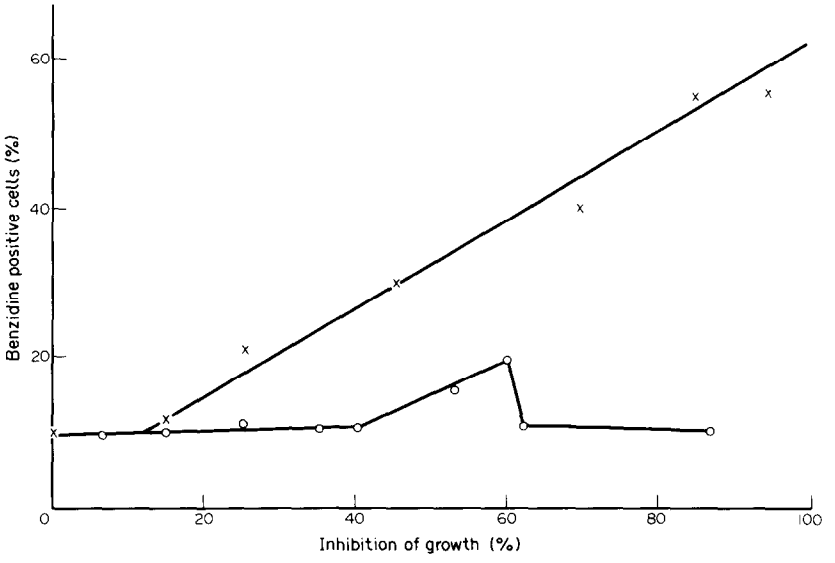

Fig. 6. Relationship between growth inhibition of K562 cells and induction of benzidine-positive cells by II(×) and IV(○) after 4 days in culture. The results are an average of 4 determinations which did not differ by more than 10%.

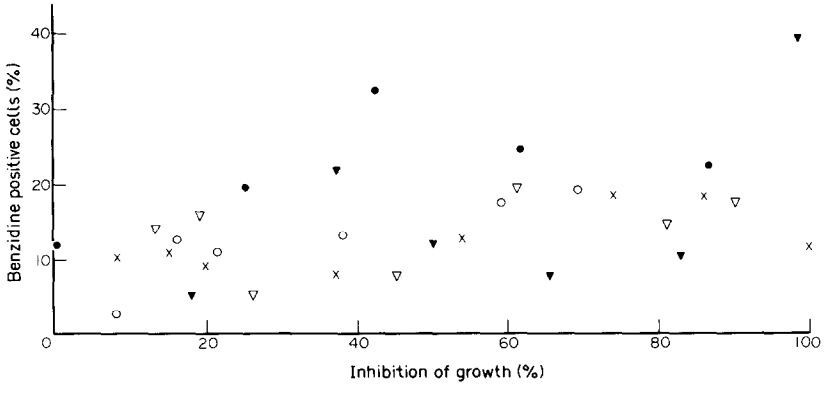

Fig. 7. Relationship between growth inhibition of K562 cells and induction of benzidine-positive cells by V(▼), VI(●) and chlorambucil (▽) after 4 days in culture. The results are an average of 3 determinations, and do not deviate significantly from control untreated culture.

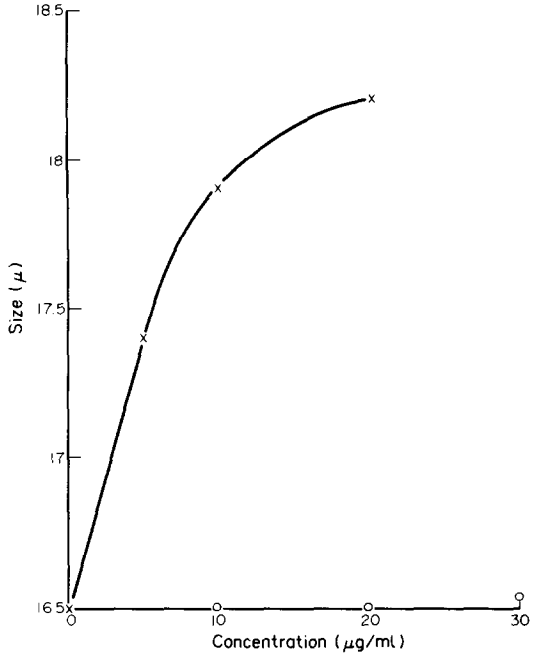

Fig. 8. Effect of I(×) and II(○) on the size of K562 cells 4 days after drug treatment. Cell size was determined by means of a Coulter electronic particle counter.

are experiments on the induction of benzidine-positive cells in newborn calf serum. Although the growth inhibitory effect of both I and II is greater in medium containing newborn serum, I is a much less effective inducer of erythroid differentiation in such media. The tenfold difference in sensitivity to I and II still remains, although the ID_{50}s are both lower (0.6 and 6.5 μg/ml respectively).

Treatment of K562 cells with I causes an increase in cell size which is dose related (Fig. 8). Agents not effective in the induction of haemoglobin synthesis, but which cause generalized toxicity, such as that associated with II have no effect on cell size. The cells staining for haemoglobin are larger than the non-staining cells.

DISCUSSION

Exposure of K562 leukaemia cells to a number of cytotoxic agents and hemin has been shown to induce the accumulation of haemoglobin, a functional property of differentiated cells [9, 13]. Most studies show haemoglobin accumulation 3 to 4 days after treatment with inducing agent. In all cases the haemoglobin synthesized is embryonic rather than adult suggesting that K562 cells must have been derived from an extremely immature progenitor [14]. This study shows that of a group of imidazotetrazinones and alkyltriazenes only the methyl analogues (I and III) are effective in the induction of haemoglobin accumulation in K562 cells, although all drugs are capable of growth inhibition.

Although 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (I) is a much more potent antitumour agent against the TLX5 lymphoma than the corresponding ethyl derivative (II) [6], no difference in cytotoxicity of the corresponding monoalkyl derivatives against the TLX5 has been observed *in vitro*; the ID_{50} being 20 μg/ml for both derivatives against both sensitive and resistant tumours. In contrast the human erythroleukaemia cell line K562 is highly discriminatory *in vitro*, the respective ID_{50}s being 3 and 28 μg/ml for the methyl and ethyl derivatives. Similarly the monomethyltriazene (III) is much more toxic than the ethyl derivative (IV) towards the K562 (0.85 and 32 μg/ml, respectively), although in a bioassay system with TLX5 lymphoma, monomethyl and monomethyltriazenes were found to have similar toxicity [15]. The reason for this difference between the cell lines is not known.

Besides being a more potent inhibitor of the growth of K562 I is also a more potent inducer of differentiation-associated properties in this cell line. Induction of benzidine positive cells by I is, however, only seen in medium containing foetal calf serum. Rowley *et al.* [9] have also observed a much higher percentage of benzidine-positive cells in medium containing foetal calf serum, although the agent(s) responsible for the difference in the two types of sera is not known. Growth inhibition for both I and II is greater in newborn calf serum (ID_{50} 0.6 and 6.5 μg/ml respectively) than in foetal calf serum, although the difference in potency of the two agents is still maintained.

Treatment of K562 cells with I at concentrations which are effective in inducing haemoglobin synthesis causes an increase in cell size of about 2μ. This size increase appears to be related to the differentiative capacity of I, since it is not observed with II even at concentrations which cause a similar effect on growth. The increase in size is also observed in K562 cells induced to differentiate with adriamycin [13].

Recent studies suggest that DNA methylation is a key element in the hierarchy of control mechanisms that govern gene function and differentiation [16]. The DNA from spontaneous animal tumours [17] and of some human cancers [18] is hypomethylated when compared with their normal counterparts. DNA methylation, induced by *N*-methyl compounds, may be responsible for the induction of erythroid differentiation of K562 cells. Induction of morphological differentiation in cultured mouse neuroblastoma cells by a series of *N*-alkyl-*N*-nitrosoureas, which can both alkylate and carbamoylate cellular components appears to be related not to carbamoylation, but to alkylation [8]. Biochemical studies on this group of imidazotetrazines [19] suggest that carbamoylation reactions do not occur, and that the effects are probably mediated through a triazene metabonate, which could alkylate cellular macromolecules. However, DNA synthesized during the induction of differentiation of Friend erythroleukaemic cells by dimethylsulphoxide is hypomethylated [20] and differentiation induced by 5-azacytidine and 5-aza-2′-deoxycytidine is also accompanied by a dose-dependent decrease of DNA methyltransferase activity and synthesis of undermethylated DNA [21]. It is possible that methylation of DNA by *N*-methyl compounds may impair the action of DNA methylating enzymes, which are

sequence specific enzymes. It has been shown that modification of DNA with NMU impairs the ability of restriction endonucleases to cleave this substrate [22] suggesting that the modification of DNA by alkylating agents may also affect the action of other proteins recognizing specific DNA sequences. Some alkylating carcinogens such as MNNG inhibit DNA methylation as a direct result of reaction with DNA methylase protein [23].

Although a number of inducers of differentiation are cytotoxic a lack of correlation between cytotoxicity and differentiation induction of HL-60 cells has been observed for the anthracycline antibiotics [24]. Although I causes growth inhibition at the concentrations which are effective in the induction of haemoglobin synthesis, viability is still maintained at the time differentiation occurs. From the slope of the line in Fig. 5 it appears that at least half of the growth inhibition obtained with I arises from terminal differentiation of leukaemic cells to forms with no further proliferative potential. Drugs with such potential could provide an alternative approach to cancer treatment from cytotoxic chemotherapy.

Acknowledgement—This work has been supported by a grant from the Cancer Research Campaign.

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