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ANTITUMOUR IMIDAZOTETRAZINES—X

EFFECT OF 8-CARBAMOYL-3-METHYLIMIDAZO[5,1-d]-1,2,3,5-TETRAZIN-4-(3H)-ONE (CCRG 81045; M & B 39831; NSC 362856) ON DNA METHYLATION DURING INDUCTION OF HAEMOGLOBIN SYNTHESIS IN HUMAN LEUKAEMIA CELL LINE K562

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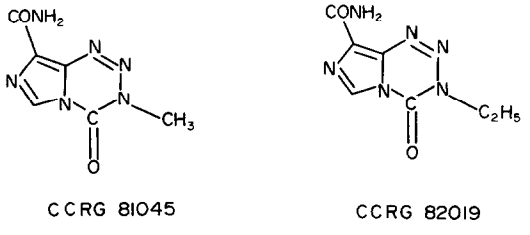
Abstract—Treatment of K562 human erythroleukaemia cells with 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (CCRG 81045) caused a concentration-dependent increase in the number of cells producing haemoglobin after 3 days of treatment. The ethyl analogue (CCRG 82019) was inactive in the induction of erythroid characteristics. The concentration of 5-methyl-cytosine in the DNA of CCRG 81045 treated cells decreased 3 days after treatment, and was directly proportional to the number of benzidine-positive cells in the cultures, suggesting a direct correlation between hypomethylation of DNA and the induction of haemoglobin synthesis. Although the mechanism of this drug-induced hypomethylation of DNA is not known, the methyl analogue (CCRG 81045) also appeared to reduce template activity of isolated DNA to a greater extent than the ethyl analogue, suggesting that the extent or position of alkylation of DNA bases be important in inhibiting DNA-recognition enzymes.

A number of chemotherapeutic agents have been shown to induce differentiation of tumour cells to more mature, functional cell types, which have lost their proliferative potential. The human erythroleukaemia cell line K562 has been reported to synthesize embryonic and foetal haemoglobins in response to haemin [1] as well as to a variety of synthetic chemicals, many of which are known to induce erythroid differentiation in Friend murine erythroleukaemia cells [2]. This cell line was originally established from a patient with chronic myeloid leukaemia in the acute phase [3] and is thought to represent a stem cell precursor of the myeloid lineage. In addition to erythroid differentiation there is increasing evidence of both granulopoietic and megakaryocytic differentiation in this cell line [4].

It has recently been shown that induction of haemoglobin synthesis occurs within three days after treatment of K562 cells with a methylimidazotetrazinone (CCRG 81045, Fig. 1) or with an *N*-methyltriazene, but does not occur after treatment with the corresponding *N*-ethyl analogues [5]. The frequency of induction of neurite formation in cultured mouse neuroblastoma cells by a series of alkylating carcinogens was also found to be higher with the methyl derivatives than the corresponding ethyl derivatives, although higher concentrations of the latter were used to induce growth inhibition to the same extent [6]. Indeed growth inhibition alone seems to be insufficient for the induction of haemoglobin synthesis in K562 cells, since although all members of the imidazotetrazinone series inhibited growth, only the *N*-methyl compound caused an alteration in gene expression [5]. 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide, a compound related to CCRG 81045, also caused the expression of biochemical differentiated function in mouse neuroblastoma cells in culture [7].



CCRG 81045 CCRG 82019

Fig. 1. Structures of chemicals used in this study.

Induction of differentiation of Friend erythroleukaemia cells by both 5-azacytidine, 5-aza-2'-deoxycytidine [8] and L-ethionine [9] is accompanied by the synthesis of markedly undermethylated DNA. A number of other experiments have suggested a role for DNA methylation in regulating or blocking transcription of DNA. Studies comparing the 5-methylcytosine content of DNA from cells in normal and neoplastic regions of the livers of carcinogen treated rats indicated that cells which had undergone marked phenotypic alteration contained extensively hypomethylated DNA [10]. Two alkylating carcinogens, *N*-methyl-*N*-nitrosourea (MNU) [11] and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) [12], which probably alkylate DNA in a manner similar to that expected for CCRG 81045 [13, 14], caused extensive hypomethylation in the DNA of human Raji lymphoblast cells two days after drug addition. The lower level of methylation persisted in the cell cycles following treatment with the carcinogens. These studies prompted the examination of the extent of enzymatic methylation of DNA in

K562 cells induced to synthesize haemoglobin after treatment with CCRG 81045.

MATERIALS AND METHODS

[5-^{3}H]Cytidine (sp. act. 28.6 Ci mmol^{-1}), 5-[methyl-^{3}H] thymidine (sp. act. 5 Ci mmol^{-1}), *S*-adenosyl-L-[methyl-^{3}H] methionine (sp. act. 68 Ci mmol^{-1}), [6-^{3}H] uridine (sp. act. 20 Ci mmol^{-1}) and eukaryotic DNA methylase were purchased from Amersham International, Bucks. Tissue culture medium and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). CCRG 81045 and CCRG 82019 were synthesized by May and Baker Ltd. (Dagenham, Essex). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset).

Cell culture conditions. Cells were maintained at 37° in RPMI 1640 medium containing 10% foetal calf serum 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 haemoglobin synthesis in K562 cells [2]. Haemoglobin synthesis was scored by benzidine staining as previously described [5].

Isotope incorporation experiments. K562 cells at an initial cell density of 4×10^4 cells ml^{-1} were incubated with the appropriate drug concentrations as indicated in Table 3. At daily intervals 1 ml of cell suspension was pulsed with 2.5 μCi of 5-[methyl-^{3}H]-thymidine and 5 μCi of [5-^{3}H] cytidine for 60 or 120 min, respectively. Incorporation of radiolabel into DNA, RNA and the acid-soluble pool was determined as described previously [15]. Pulse labelling was also carried out for a 60 min period with [5-^{3}H]-uridine or L-[methyl-^{3}H] methionine (both at 5 μCi ml^{-1}). Incorporation of radioactivity into acid-insoluble material was determined by filtering the cell suspension through a Whatman GF/C glass fibre disc with 0.9% NaCl, followed by 10 volumes of absolute ethanol. The discs were dried at 70° for 1 hr and the radioactivity was determined in a toluene, PPO/POPOP scintillation mix.

Isolation and hydrolysis of nucleic acids. This was carried out by a method similar to that of Kredich and Hershfield [16]. After drug treatment cells were pelleted by centrifugation and washed once with 0.9% NaCl. The cells were dissociated with 0.5 ml of cold 1 M perchloric acid and the perchloric acid-insoluble material was washed with 70% and then absolute ethanol, dried in a stream of nitrogen and suspended in 0.7 ml of 0.1 M Tris–HCl, pH 7.6, containing 0.5% sodium dodecyl sulphate, 20 mM Na_2 EDTA and nuclease free Pronase at 1 mg/ml. After overnight incubation at 37° the solution was extracted three-times with water-saturated phenol and excess phenol was removed from the aqueous layer by extraction with diethyl ether. The nucleic acids were precipitated with 2 vol. of cold ethanol, collected by centrifugation, washed with 70% and then absolute ethanol, dried in a nitrogen stream and dissolved in 0.3 ml of 0.25 M NaOH. The solution was incubated at 37° for 18 hr to hydrolyse RNA and the DNA was precipitated by adding 80 μl of cold 5 M perchloric acid. The DNA was washed with 70% and then absolute ethanol, dried and hydrolysed by heating at 180° for 30 min in 200 μl of 90% (wt/vol.) formic acid in a sealed tube. The formic acid was removed in a stream of nitrogen and the residue was dissolved in 200 μl of water and analysed for base composition by high-performance liquid chromatography on a Partisil 10 SCX column (0.6 × 25 cm, Whatman) eluted isocratically with 0.07 M ammonium formate, pH 3.2, at room temperature in an Altex h.p.l.c. system. Bases were identified relative to the elution of authentic compounds and their quantity determined by measurement of the base peak area at 280 nm/base extinction coefficient at 280 nm at pH 3.2 using a Waters LC Spectrometer. The 5-methylcytosine content of the samples was determined in triplicate and at least three separate determinations were made for each concentration of drug and day of incubation. Control experiments with cytosine and 5-methylcytosine showed no demethylation under the conditions of acid hydrolysis. For labelling of DNA 10 μCi of [6-^{3}H] uridine was added to 50 ml of the culture medium 24 hr prior to harvesting. DNA concentration was determined according to the method of Gold and Shochat [17]. The concentration of *S*-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-homocysteine (SAH) in the perchloric acid-soluble fraction of the cell was determined, after neutralisation with KOH and removal of the insoluble $KClO_4$, on a Partisil 10 SCX column eluted isocratically with 0.5 M ammonium formate, pH 4.0, at room temperature and detected by the u.v. absorption at 254 nm according to the method of Zappia *et al.* [18].

The ability of isolated DNA to support DNA-dependent RNA synthesis was assayed using *Escherichia coli* RNA polymerase according to the method of Marushige and Marushige [19].

RESULTS

Treatment of K562 human erythroleukaemia cells with CCRG 81045 caused a concentration and time-dependent increase in the number of benzidine-positive cells in the cultures after 3 days of treatment (Fig. 2). In contrast, CCRG 82019 did not cause the formation of benzidine-positive cells, even at concentrations having an equivalent effect on cell growth to those of CCRG 81045 [5]. In cells treated with CCRG 81045, the concentration of 5-methylcytosine in newly replicated DNA decreased 3 days after drug treatment in a dose-dependent manner (Table 1). Thus in cells grown in the presence of 73.5 μM of CCRG 81045, which is the maximum concentration capable of inducing haemoglobin synthesis without extensive cytotoxicity, the DNA is about 40% less methylated as compared with controls. This figure is very similar to that obtained with 10 μg/ml MNNG on human Raji cells [12]. The decrease in methylation cannot be attributed to a lower extent of DNA synthesis in treated cells, since the extent of enzymatic methylation was calculated on the basis of the conversion of [6-^{3}H] uridine into 3-methylcytosine. In fact, the incorporation of 5(methyl-^{3}H) thymidine per cell does not alter substantially during the duration of the treatment in

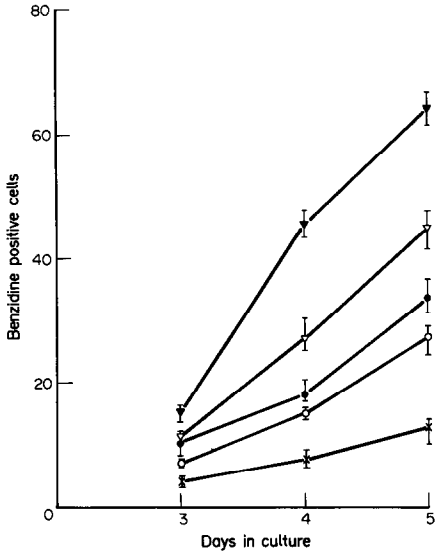


Fig. 2. Kinetics of appearance of benzidine-positive K562 cells cultured in the absence (×) or in the presence of 4.9 (○), 24.5 (●), 36.8 (▽) or 73.5 (▼) μM of CCRG 81045. The percentage benzidine-positive cells was evaluated on a minimum of 200 cells counted in a haemocytometer. Each point is the mean of four experiments and is shown with the S.E.

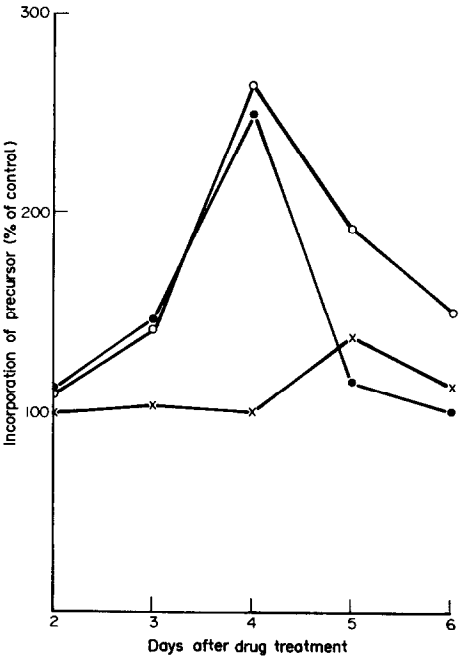


Fig. 3. Effect of 36.8 μM of CCRG 81045 on the incorporation of 5-[methyl-^{3}H] thymidine (×), [5-^{3}H] uridine (●) and L-[methyl-^{3}H] methionine (○) into acid-insoluble material of K562 cells. Results are calculated as the incorporation per cell and are expressed as a percentage of control cells cultivated without the drug. Each point is the mean of five experiments which were carried out in triplicate. The variation between individual experiments was not greater than 10%. Control cultures incorporated/10^5 cells 27,300 cpm of [methyl-^{3}H] thymidine, 4880 cpm of [5-^{3}H] uridine and 4100 cpm of L-[methyl-^{3}H] methionine.

contrast with the incorporation of [5-^{3}H] uridine and L-(methyl-^{3}H) methionine which is maximal at day 4 (Fig. 3). The 5-methylcytosine content of the DNA of cells treated with CCRG 81045 is directly proportional to the number of benzidine-positive cells in the cultures (Fig. 4). This suggests a direct correlation between the hypomethylation of the DNA and the induction of the differentiated phenotype in K562 cells. In contrast CCRG 82019, which has no effect on phenotypic expression at the concentrations employed, has no effect on the 5-methylcytosine content of the DNA. Treatment with either CCRG 81045 or CCRG 82019 has no effect on the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine (results not shown).

The DNA of K562 cells isolated after treatment with various concentrations of CCRG 81045 and CCRG 82019 was also examined for its ability to support DNA-dependent RNA synthesis catalyzed by exogeneously added *Escherichia coli* RNA polymerase. As shown in Table 2, template activity reaches a maximum between 2 and 3 days after drug treatment, and thereafter decreases towards control values. The increased transcriptional activity of isolated DNA precedes the appearance of the differentiated phenotype. When calf thymus DNA is treated with various concentrations of CCRG 81045 or CCRG 82019 and examined for its ability to support DNA-dependent RNA synthesis inhibition

Table 1. Extent of enzymatic methylation of DNA in K562 cells in the presence of CCRG 81045 and CCRG 82019*

Treatment	Day 1	2	3	4	5
None	3.4 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.5 ± 0.1
CCRG 81045 24.5 μM	3.9 ± 0.4	3.5 ± 0.1	2.85 ± 0.1	3.0 ± 0.1	3.2 ± 0.1
CCRG 81045 49.0 μM	3.9 ± 0.4	3.2 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
CCRG 81045 73.5 μM	3.5 ± 0.3	3.4 ± 0.2	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
CCRG 82019 120 μM	3.7 ± 0.5	3.1 ± 0.1	3.8 ± 0.2	3.7 ± 0.1	3.5 ± 0.1
CCRG 82019 240 μM	3.6 ± 0.4	3.8 ± 0.3	3.3 ± 0.2	3.6 ± 0.2	3.7 ± 0.3

* Results are means of at least three determinations for each time point, and are given ± S.E.M.

The figures refer to per cent of total cytosines methylated $= \frac{5MC}{C + 5MC} \times 100$.

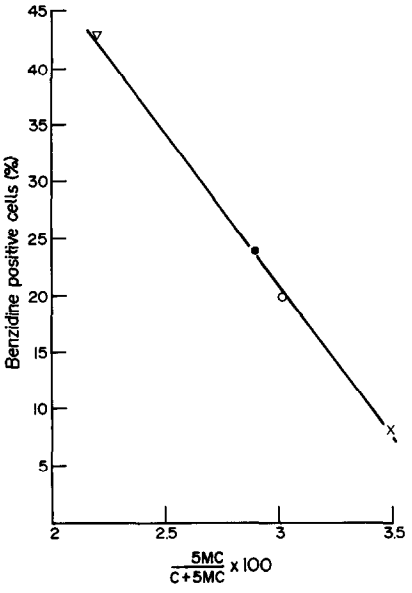


Fig. 4. Relationship between 5-methylcytosine content of DNA of K562 cells 4 days after treatment with 0 (×), 24.5 (○), 49 (●) and 73.5 (▽) μM of CCRG 81045 and the induction of benzidine-positive cells.

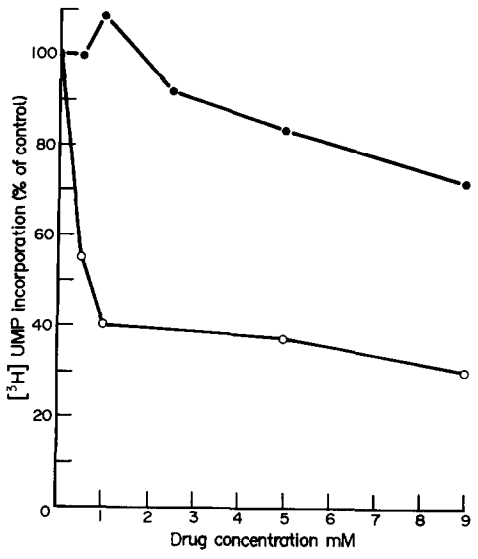


Fig. 5. Template activity of DNA after treatment with various concentrations of CCRG 81045 (○) or CCRG 82019 (●). Calf thymus DNA (300 μg/ml) in 10 mM Tris–HCl, pH 8.0, was treated with various concentrations of each drug at 23–24° for 60 min. The dialysed mixture was then examined for its ability to support DNA-dependent RNA synthesis.

is observed, which increases with increasing concentrations of both drugs (Fig. 5). However, at equimolar concentrations CCRG 81045 inhibits template activity to a greater extent than CCRG 82019. Thus the transcriptional activity of DNA isolated from cells treated with CCRG 81045 will be a balance between an increase arising from hypomethylation of the DNA and a decrease possibly resulting from the formation of 7-alkylguanine and alkyl phosphotriesters in DNA [19]. However, the concentration of drugs required for DNA modification *in vitro* is approximately 1000-fold greater than is required for effects in the intact cell.

One possibility for the hypomethylation of DNA by drugs which can alkylate guanine in the 0–6 position is mispairing during replication and the subsequent insertion of thymine in place of cytosine in newly replicated DNA [20]. To investigate this possibility the ratio of the incorporation of 5-(methyl-^{3}H) thymidine to [5-^{3}H] cytidine has been measured at intervals after treatment of K562 cells with CCRG 81045 and CCRG 82019. The ratio expressed as a percentage of a control culture is shown in Table 3. It can be seen that the ratio significantly increases in cultures treated with CCRG 81045 after 3 days, whereas CCRG 82019 was much less effective in causing mispairing. Neither drug affected the pool size of either nucleoside. Thus one possibility for the decreased 5-methylcytosine content of newly replicated DNA is a decreased cytosine content.

DISCUSSION

The antitumour imidazotetrazinone, mitozolomide, undergoes decomposition in aqueous solution at an alkaline pH to give the monochloroethyltriazene MCTIC [21]. Evidence has been presented that similar reactions occur within the cell [22]. By analogy the methyl analogue CCRG 81045 would yield the methyltriazene, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide, which might be expected to methylate DNA at the 0–6 position of

Table 2. Effect of CCRG 81045 and CCRG 82019 on the template activity of DNA isolated from K562 cells

	[^{3}H] UMP incorporated (% of control)			
	Day			
Treatment (μM)	2	3	4	5
CCRG 81045 24.5	119 ± 10	123 ± 12	113 ± 8	112 ± 10
CCRG 81045 49	122 ± 8	148 ± 18	132 ± 12	100 ± 10
CCRG 81045 73.5	171 ± 14	157 ± 15	144 ± 12	118 ± 10
CCRG 82019 120	129 ± 10	110 ± 10	104 ± 8	43 ± 10

The results (±S.E.M.) are means of three experiments carried out in duplicate. Controls incorporated 6000 cpm of [^{3}H]UMP.

Table 3. Effect of CCRG 81045 and CCRG 82019 on the incorporation of 5-[methyl-^{3}H]thymidine (T) and [5-^{3}H] cytidine (C) into newly synthesized DNA

Treatment (μM)	T/C (% of control)* Day 1	Day 2	Day 3
CCRG 81045 24.5	112 ± 10	127 ± 7	144 ± 8
CCRG 81045 49.0	112 ± 10	139 ± 9	161 ± 10
CCRG 81045 73.5	132 ± 10	131 ± 8	183 ± 10
CCRG 82019 120	94 ± 15	131 ± 20	96 ± 8
CCRG 82019 240	100 ± 6	113 ± 3	127 ± 15

* Results (±S.E.M.) are means of three experiments carried out in duplicate. Control cells incorporated 10,000 dpm of T and 3500 dpm of C.

guanine. Indeed CCRG 81045 shows greater cytotoxicity to cells deficient in the repair of 0–6 guanine lesions (Mer$^-$) than to repair proficient cells (Mer$^+$), and in this respect resembles mitozolomide (Horgan and Tisdale, unpublished results). Since O^6-methylguanine mispairs with thymine in place of cytosine it has been suggested [20] that the critical result of such a transition is the loss of cytosine residues, which may otherwise be potential sites of enzymatic methylation at the C-5 position. Some support for this hypothesis is provided in the present experiments where the incorporation of [5-^{3}H] cytidine into acid-insoluble material of cells treated with CCRG 81045 decreases as compared with the incorporation of 5-(methyl-^{3}H) thymidine.

However, alkylation of DNA may also impair the action of proteins which recognise specific sequences on a DNA molecule. Modification of DNA with MNU has been shown to impair the ability of restriction endonucleases to cleave this substrate [23]. Since enzymatic methylation is catalysed by sequence-specific enzymes [24], alkylation of a parental strand of DNA may result in an aberrant methylation pattern in the progeny strand [11, 12]. DNA methylases do not bind to a methyl-accepting polymer in which the N-7 position of guanine is alkylated [25]. This suggests that N-7 alkylation of guanine residues by CCRG 81045 may be more important in the alteration in phenotypic expression than 0–6 alkylation. Presumably the ability of isolated DNA from cells treated with CCRG 81045 to act as a substrate for DNA methylase *in vitro* is due to removal of N-7 alkylated bases from the DNA during the isolation procedure.

In-vitro alkylation of DNA by CCRG 81045 reduces its ability to support RNA synthesis catalysed by exogeneously added RNA polymerase. In contrast, at the same concentrations CCRG 82019 is much less effective in reducing the template activity of DNA. This result is similar to that obtained with calf thymus DNA treated MNU and *N*-ethyl-*N*-nitrosourea (ENU) [19] and arises from the greater reactivity of the former towards DNA. At equimolar concentrations MNU reduces the ability to support RNA synthesis more than ENU. ENU has been reported to be more reactive to the O^6-position of DNA guanine than MNU [26]. This difference is, however, small (12% of the total alkylated products for ENU vs 6% for MNU). However, MNU is 40-times more reactive to chromatin DNA than ENU [27], although the rate of removal of O^6-ethylguanine has been shown to be 3.6-times slower than the rate of removal of O^6-methylguanine [28]. However, the extent of N-7 methylation of guanine by MNU is 6-times greater than ENU which produces mainly phosphotriester linkages [29]. This again suggests that N-7 alkylation of guanine may be the important reaction, impairing the ability of DNA-binding proteins to recognize particular sections of DNA, and that this loss of sequence recognition may be an important factor determining the selectivity of these type of agents *in vivo*.

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