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ANTITUMOUR IMIDAZOTETRAZINES—XXIV

GROWTH SUPPRESSION BY DNA FROM CELLS TREATED WITH IMIDAZOTETRAZINONES

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Abstract—Transfection of a murine colon adenocarcinoma cell line (MAC13) with DNA extracted from GM892 or Raji cells previously treated with either the methyl- (temozolomide) or ethyl- (CCRG82019) imidazotetrazinones caused a dose-related suppression of cell growth. The effect was proportional to the concentration of DNA transfected and the time of incubation of the donor cell lines with the drugs. It was not shown with X-irradiated DNA suggesting that the effect did not arise from non-specific damage to the DNA. Transfection of MAC13 cells with DNA extracted from GM892 cells was more effective in inhibiting growth than DNA from Raji cells, and temozolomide treated cellular DNA was a more potent growth inhibitor than that from CCRG 82019 treated cells. For both agents the growth inhibitory effect was most marked with DNA extracted 6 hr after drug addition and thereafter the effect decreased up to 24 hr after drug addition. This suggests that the growth inhibitory effect is due to a repairable lesion, and that the terminal mechanism of action of these agents involves targets after DNA.

In the series of 3-alkyl substituted imidazotetrazinones, strict structural requirements are required for the alkyl group to exert effective antitumour activity. Thus, while 3-(2-chloroethyl)- (mitozolomide) and 3-methyl- (temozolomide) imidazotetrazinones are effective antitumour agents, the presence of a 3-ethyl-(CCRG 82019), or higher alkyl substituents, leads to a loss of cytotoxic potency *in vitro* [1] and antitumour activity *in vivo* [2]. A similar structure–activity relationship is shown in the series of antitumour triazenes and nitrosoureas [3]. The antitumour activity of drugs of these types has been linked to alkylation of the O-6 position of guanine in DNA. Thus, cells deficient in the capacity to repair lesions at the O-6 position of guanine (Mer^-) show an increased sensitivity to both a monomethyl or monochloroethyltriazene, compared with a repair proficient cell line (Mer^+) [4]. However, no difference in toxicity between a Mer^- and Mer^+ cell line was observed with an ethyltriazene, suggesting that O^6-alkylation of guanine may not be important for the cytotoxicity of the ethyl derivative. In the case of the imidazotetrazinones, depletion of the repair capacity of a Mer^+ cell line with the free base O^6-methylguanine resulted in an increased sensitivity towards both mitozolomide and temozolomide, but not the ethyl derivative CCRG 82019.

Alkylation of guanine with a chloroethyl group leads to interstrand cross-linking arising from a secondary reaction of the chloroethyl group with the N-3 position of a cytosine residue in the opposite DNA strand after migration to the N-1 position of guanine [6]. However, it is difficult to see how a methyl group at the O-6 position of guanine could lead to cytotoxicity, since cross-linking is chemically impossible. While mutation induction correlates well with the production of O^6-methylguanine in DNA, there is a less clear correlation between cytotoxicity and O^6-methylguanine production. Thus while some authors have shown a correlation between unrepaired O^6-methylguanine and cytotoxicity [7], others have failed to find such a correlation [8]. Some workers have suggested that adducts at the O-6 atom of guanine in DNA are not potentially cytotoxic lesions and that defects other than lack of methyltransferase is responsible for the sensitivity of Mer^- cells to killing by alkylating agents [9]. In order to investigate the potential mechanisms responsible for cytotoxicity we have determined the ability of alkylated DNA to directly influence the growth of a cell line after direct transfection.

MATERIALS AND METHODS

Tissue culture medium and foetal calf serum were purchased from Gibco Ltd (Paisley, U.K.). Temozolomide and CCRG 82019 were synthesized by May and Baker Ltd (Dagenham, U.K.) and all other chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.).

Cell culture. Cell lines Raji (human Burkitts lymphoma, GM892 (human lymphoblastoma) and MAC13 (murine colon adenocarcinoma) were maintained in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. Drugs were dissolved in dimethylsulphoxide (DMSO), such that the final concentration of DMSO in the culture medium did not exceed 0.4%. Both Raji and GM892 cells were exposed to equitoxic concentrations of the drugs at the ID_{50} values (Table 1) for either 6, 12 or 24 hr and the DNA was extracted by the method of Warren [10]. Cells were isolated from culture medium by centrifugation, washed with 0.9% NaCl, treated with 6% 4-aminosalicylic acid and lysed with 10% SDS. After

Table 1. Sensitivity of cell lines to imidazotetrazinones and O^6-alkylguanine-DNA alkyltransferase activity (O^6MeGMT)

Cell line	ID_{50} (μM)*		O^6MeGMT (fmol/mg protein)
	Temozolomide	CCRG 82019	
GM892	10 ± 7	229 ± 20	10 ± 5
Raji	206 ± 20	360 ± 30	634 ± 80
MAC13	77 ± 10	>1000	44 ± 4

Results are expressed as means ± SE from the average of at least three experiments.
* Concentration required to produce 50% inhibition of cell growth.

extraction with phenol reagent the DNA was precipitated with 2-ethoxyethanol, washed extensively with 70% ethanol followed by absolute ethanol, and allowed to dry overnight.

Transfection of DNA into eukaryotic cells. The day before transfection, MAC13 cells were split into individual flasks at a concentration of 5×10^4cells per flask containing 9 mL of new RPMI 1640 medium, plus 10% foetal calf serum and allowed to adhere overnight. The DNA for transfection was sterilized by precipitation with two volumes of absolute ethanol and air dried in a sterile hood. MAC13 cells were treated with 30 μg of DNA co-precipitated with calcium phosphate, which was distributed evenly over the surface of the cells and gently agitated to mix the precipitate and the medium. The cells were then incubated for 5 hr, after which time the medium was removed and the cells were washed twice with 5 mL of 1 ×phosphate buffered saline. The cells were then fed with 10 mL of complete medium, and incubated under an atmosphere of 5% CO_2 in air for 3 to 4 days, when the cells were removed by trypsin treatment, and the cell number was enumerated with a Coulter electronic particle counter, model D.

For the experiments with X-irradiated DNA, cells were transfected with 30 μg of calf thymus DNA which had previously been irradiated with various doses of radiation from 72 to 400 rads.

Statistical analysis. All results are expressed as mean ± SE for at least three separate determinations. Differences have been analysed statistically using Student's *t*-test.

RESULTS

The concentrations of temozolomide and CCRG 82019 required to produce a 50% inhibition of growth of the three cell lines is shown in Table 1. The level of response appears to be related to the concentration of the repair enzyme O^6-methylguanine methyltransferase (O^6MeGMT) in a particular cell line. Thus, GM892 cells which are highly sensitive to the cytotoxic effect of temozolomide, have low levels of the repair protein, while Raji cells which are insensitive, have high levels of the repair protein, and an intermediate level is found in the MAC13 cell line.

In order to try to understand the cellular reactions responsible for cytotoxicity after treatment with the imidazotetrazinones, cellular DNA extracted from GM892 or Raji cells has been transfected into MAC13 cells using the calcium phosphate precipitation technique. Transfection of MAC13 cells with DNA from GM892 or Raji cells, which have only been exposed to the solvent, does cause a reduction in cellular proliferation compared with untreated controls (Fig. 1), and therefore results with DNA obtained from drug treated cells have been compared with those obtained with solvent control. For both Raji or GM892 cells treated with either temozolomide or CCRG 82019 at equitoxic concentrations, the DNA when transfected into MAC13 cells, causes an inhibition of proliferation. Incubation of MAC13 cells with DNA from either cell line in the absence of calcium phosphate had no effect on the subsequent proliferation. The results in Fig. 1 show that the effect is dependent on the length of time the donor cells have been incubated with drug prior to DNA extraction. Thus, for DNA extracted from both GM892 cells (Fig. 1A) or Raji cells (Fig. 1B), the effect is most pronounced with DNA extracted 6 hr after drug addition, and becomes progressively less so 12 and 24 hr after drug addition. Transfection of MAC13 cells with DNA from GM892 cells treated with either temozolomide or CCRG82019 has a more profound effect on the subsequent growth than that extracted from Raji cells, despite the higher concentrations of drugs to which these cells were exposed (Table 1). For temozolomide-treated cells at 6 hr both DNA from GM892 and Raji cells produces a highly significant ($P < 0.001$ and $P < 0.005$, respectively) inhibition of the growth of MAC13 cells. However, at 12 and 24 hr only, GM892 DNA still significantly ($P < 0.005$ and $P < 0.05$, respectively) inhibits the growth of MAC13 cells. Similarly for CCRG 82019-treated cells at 6 hr, both DNA from GM892 and Raji cells produces a significant ($P < 0.001$ and $P < 0.05$, respectively) inhibition of growth of MAC13 cells, while at 12 and 24 hr only GM892 DNA still significantly inhibits growth ($P < 0.005$ and $P < 0.05$, respectively).

DNA extracted from cells treated with CCRG 82019 appears to be a less potent inhibitor of the growth of MAC13 cells than that extracted from cells treated with temozolomide. Thus at 6 hr, DNA from both GM892 and Raji cells exposed to temozolomide is a more potent ($P < 0.05$ and

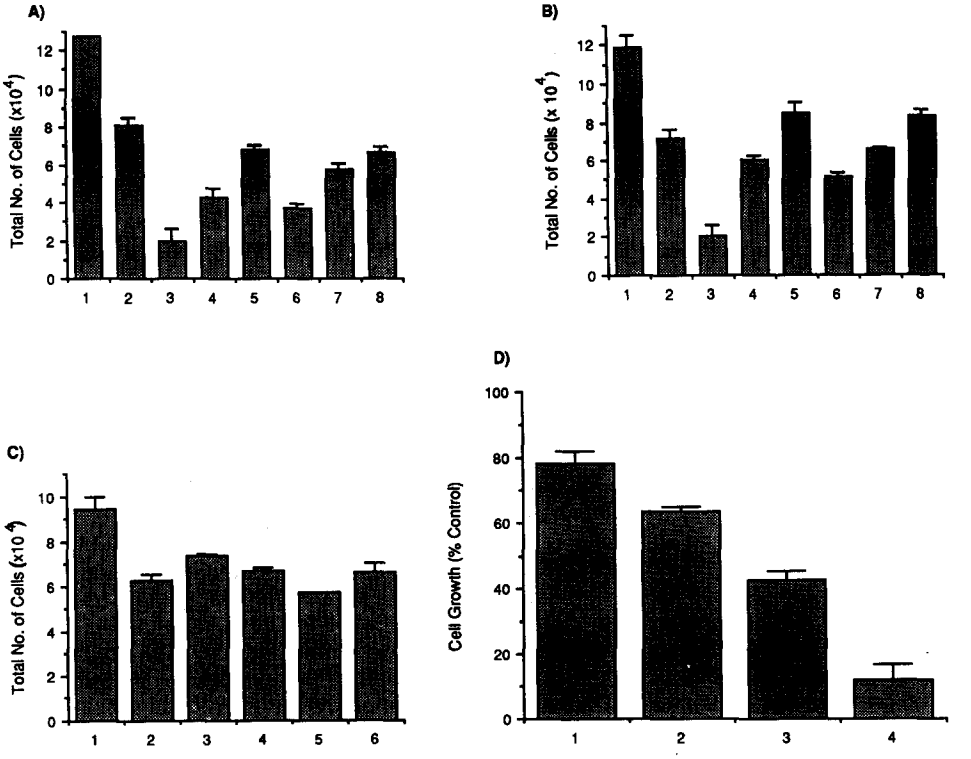

Fig. 1. Effect on growth of MAC13 cells of transfection with DNA isolated from (A) GM892 and (B) Raji cells or (C) calf thymus DNA previously exposed to various doses of X-irradiation and (D) calf thymus DNA treated with 10 mM temozolomide *in vitro*. In (A) and (B) cells were transfected with calcium phosphate co-precipitated DNA (30 μg) isolated from solvent controls cells (2) or cells which had been exposed to temozolomide (3–5) or CCRG 82019 (6–8) at the ID_{50} concentrations for 6 (3, 6), 12 (4, 7) and 24 hr (5, 8) and the effect on subsequent cellular proliferation was determined as compared with non-transfected cells (1). In (C) cell growth in the absence of DNA (1) was compared with that in cells transfected with calf thymus DNA, which was either untreated (2) or subjected to 72 (3), 100 (4), 200 (5) or 400 (6) rads of X-irradiation, while in D cells were transfected with 25 (1), 50 (2), 100 (3) or 150 (4) μg of temozolomide treated calf thymus DNA and cell growth was compared with the equivalent amounts of non-treated calf thymus DNA.

$P < 0.005$, respectively) inhibitor of the growth of MAC13 cells than DNA from cells exposed to CCRG 82019.

For cells exposed to temozolomide for 6 hr, the growth inhibitory effect is proportional to the concentration of drug up to 500 μM (Fig. 2) and is proportional to the concentration of DNA used in the transfection assay (Fig. 3A and B). The inhibition of cell growth is not due to a non-specific effect from damaged DNA, since transfection of MAC13 cells with calf thymus DNA irradiated with up to 400 rads of X-irradiation has no effect on the subsequent growth (Fig. 1C), while transfection with calf thymus DNA previously treated with 10 mM temozolomide causes a progressive decrease in cell growth with increasing concentrations of DNA (Fig. 1D).

DISCUSSION

These results suggest that alkylation of DNA by the imidazotetrazinones is not the ultimate reaction, but that the alkylated DNA is capable of exerting other actions in the cell, which are ultimately

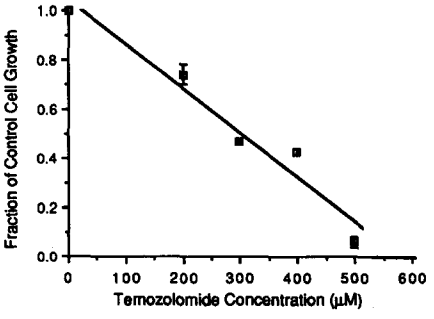

Fig. 2. The effect on growth of MAC13 cells of transfection with DNA (30 μg) isolated from Raji cells exposed to various concentrations of temozolomide for 6 hr. Results are expressed as mean ± SE relative to the effect of DNA from solvent treated control cells.

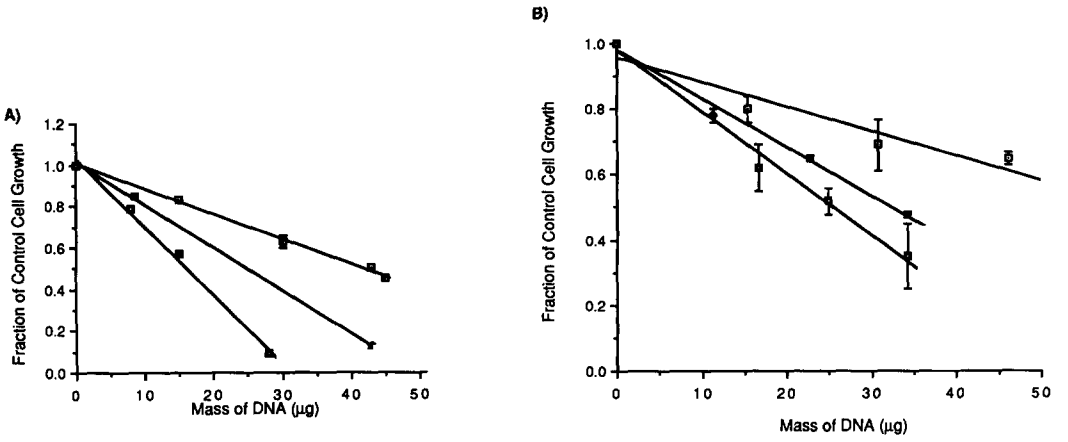

Fig. 3. Effect on growth of MAC13 cells of transfection with various concentrations of DNA isolated from Raji cells 6 hr after treatment with 200 (⊡), 400 (■) and 500 (□) μM temozolomide (A) or 192 (⊡), 385 (■) and 480 μM (□) CCRG 82019 (B).

responsible for the inhibition of cellular proliferation. The effect appears not to be due to a non-specific inhibition of proliferation by damaged DNA, e.g. by single-strand breaks since X-irradiated calf thymus DNA does not produce a similar action. However, calf thymus DNA, previously treated *in vitro* with temozolomide, when transfected into MAC13 cells produces growth inhibition in a manner similar to that from DNA extracted from cells exposed to this agent. The time course for the inhibition of growth by transfected DNA suggests a repairable lesion. The greater potency of temozolomide over CCRG 82019, and the greater effectiveness of DNA from GM892 over Raji cells correlates with the direct effect of these agents on the two cell lines. This has previously been attributed to O^6-alkylation of guanine residues, which is related to the repair capacity for this particular lesion. However, at the LD_{50} levels of drugs utilized in the present study, the repair proteins are effectively titrated out, so that there is little removal of O^6-methyguanine over a 24 hr time period [11]. This suggests that the O^6-alkylguanine lesion is not responsible for the growth inhibitory effect of the DNA.

However, two lesions found in both cell lines, which are repairable and which may contribute to the cytotoxicity of alkylated DNA are the formation of 7-alkylguanine and 3-alkyladenine. In addition, the yield of both these alkylated bases in cells exposed to temozolomide (70 and 9.2% of the total products, respectively), is much higher than in cells exposed to CCRG 82019 (24 and 4.9% of the total products, respectively) with the latter forming mainly phosphotriesters [11]. Medcalf and Lawley [12] have studied the rate of removal of alkylated bases from the DNA of *N*-methyl-*N*-nitrosourea (NMU) treated human fibroblasts and have found that removal of both of these bases occurs, but that 3-methyladenine is removed much more rapidly with a time course approximating to that of the alkylated DNA used in the transfection studies reported here. This suggests that the most likely lesion responsible for the growth inhibitory effect of the alkylated DNA is the formation of 3-alkyladenine. 3-Methyladenine has been implicated in the lethal effects of alkylating agents to bacteria [13], but its importance in eukaryotic cells has not been demonstrated.

The cellular reactions responsible for the ultimate toxicity of the transfected DNA are not known. However, it is interesting to note that DNA extracted from GM892 cells previously treated with temozolomide was capable of inhibiting the methylation of *M. lysodeiktious* DNA by purified DNA–cytosine 5-methyltransferase [14], with a time course similar to that with the transfected DNA observed in the present study. This suggests that the repairable lesion was the same in both cases. Modification of DNA by chemical carcinogens interferes with the enzymatic methylation of DNA both *in vitro* [15] and *in vivo* [16]. Although the relationship to the antiproliferative effects of these agents is not known, it is interesting to note that the antileukaemic action of 5-aza-2′-deoxycytidine has been correlated with its effects on DNA methylation [17].

Transfection of MAC13 cells with [^{3}H]thymidine labelled DNA results in an incorporation of 0.3% of the radioactivity into the cell, when 17 μg of DNA is used in the transfection experiment. It is not known whether the transfected DNA is incorporated into the murine genome, or whether the alkylated DNA is exerting its growth inhibitory effect independent of incorporation. Further studies are now being conducted to investigate this novel event.

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