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ANTITUMOUR IMIDAZOTETRAZINES—XVI
MACROMOLECULAR ALKYLATION BY 3-SUBSTITUTED IMIDAZOTETRAZINONES

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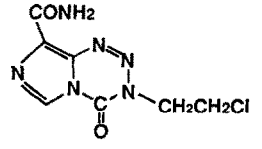
Abstract—The extent of macromolecular alkylation by three imidazotetrazinones, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3H)-one (mitozolomide) and the 3-methyl CCRG 81045) and 3-ethyl (CCRG 82019) analogues has been studied both in intact cells and with isolated DNA, RNA and protein. Towards isolated DNA and RNA CCRG 81045 was about twice as reactive as mitozolomide and 5–10-fold more reactive than CCRG 82019. Two cell lines were chosen to study macromolecular alkylation, GM892A and Raji, the latter being 10–20-fold less sensitive to mitozolomide and CCRG 81045 than the former, but only one-and-a-half-fold less sensitive to CCRG 82019. Drug uptake into both cell lines was shown to be by a rapid diffusion process with a cell medium distribution ratio not far from unity. For all three agents intracellular radioactivity became associated with macromolecules, and the level found at any time is a balance between the rate of alkylation and the rate of alkyl group removal by repair processes. Both CCRG 81045 and CCRG 82019 produced approximately the same level of alkyl groups bound to DNA, RNA and protein over a 24-hr period, whereas mitozolomide produced a greater extent of alkylation. All three agents left more alkyl groups bound to DNA and RNA in GM892A than in Raji cells, but there was no difference in the level of alkyl groups remaining bound to proteins. However, in GM892A cells the overall level of alkylation of DNA by CCRG 81045 exceeded that of CCRG 82019 only after 24 hr of drug incubation despite the twenty-fold difference in potency of these agents. These results suggest that specific base alkylations rather than total macromolecular alkylation may be more important in determining relative cytotoxicity.

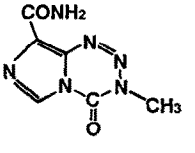
The imidazotetrazines are a new group of broad spectrum antitumour agents which are highly active against both transplantable animal tumours [1, 2] and human tumour xenografts [3]. A strict structure–activity relationship is observed for substituents in the 3-position and against the murine TLX5 lymphoma only R = CH_2CH_2Cl (Mitozolomide, CCRG 81010) and R = CH_3 (Temozolomide CCRG 81045) show activity [2]. A similar relationship is observed with the antitumour triazenes [4], and both chemical [5] and biological [6, 7] studies suggest that the imidazotetrazines may act as pro-drugs of the active triazenes with ring opening occurring *in vivo*. In addition both groups of drugs show selective toxicity to cell lines lacking O^6-methyl-guanine methyltransferase (Mer^- phenotype) [8, 9], suggesting that alkylation of DNA guanine in the O^6 position may be the primary cytotoxic event.

The cytotoxicity of mitozolomide has been correlated with interstrand cross-linking of DNA [10, 11], which probably arises after an initial alkylation at the O^6 position of guanine residues in DNA. However, CCRG 81045 is incapable of cross-link formation, but still shows antitumour activity, suggesting that binding of the alkyl group at the 3-position of the imidazotetrazine nucleus to macromolecules may be sufficient for activity. In this case it is not clear why the higher homologue (R = C_2H_5, CCRG 82019; Fig. 1) should be inactive.

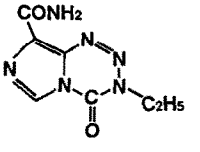
The greater carcinogenic potency of *N*-methyl-*N*-nitrosourea (MNU) in adult animals when compared with ENU has been attributed to differences in relative reactivities towards DNA and proteins [12]. In the imidazotetrazine series CCRG 81045 (R = CH_3; Fig. 1) has a greater inhibitory effect on the template activity of isolated DNA than CCRG 82019 (R = C_2H_5; Fig. 1) [13]. This suggests that the difference in reactivity of the two analogues towards cellular macromolecules may be responsible for the difference in biological activity. This study determines the extent of reaction of different 3-substituted imidazotetrazines with DNA, RNA and protein and attempts to correlate the differences in reactivity with the biological effects.



CCRG 81010
(Mitozolomide)



CCRG 81045



CCRG 82019

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

MATERIALS AND METHODS

8-Carbamoyl-3-[^{14}C]methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (sp. act. 20.2 mCi mmol^{-1}) and 8-carbamoyl-3-[^{14}C]ethylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (sp. act. 12.9 mCi mmol^{-1}) were synthesized by ICI and kindly donated for this study by May & Baker Ltd. (Dagenham, U.K.) 8-Carbamoyl-3-[^{14}C]-2-chloroethylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3H)-one (sp. act. 15.8 mCi mmol^{-1}) and the unlabelled imidazotetrazines were synthesized and donated by May & Baker Ltd. Tissue culture medium and foetal calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland). Calf thymus DNA, calf liver RNA and bovine serum albumin were purchased from Sigma Chemical Co. (Dorset, U.K.). Labelled drugs were made up in dimethyl sulphoxide (DMSO) at a concentration of 5 mg ml^{-1} and stored frozen at −20°.

In vitro *reactions of imidazotetrazinones with DNA, RNA and protein.* Radiolabelled drugs were incubated with 600 μg of DNA, RNA or bovine serum albumin in 300 μl of 50 mM KCl plus 700 μl of 50 mM Tris–HCl, pH 7.8, 10 mM EDTA and 10 mM dithiothreitol for 2 hr at 37°. The reactions with DNA and RNA were terminated by chilling on ice, adding sodium acetate to give a 2% solution, followed by precipitation of the macromolecules with 3 vol. of ice-cold absolute ethanol. The DNA and RNA were collected by centrifugation and non-reacted drug removed by two washes in ice-cold absolute ethanol. The reaction with protein was terminated by chilling the solution on ice followed by the addition of 3 ml of ice-cold 10% trichloroacetic acid. The protein was collected by centrifugation and washed and recentrifuged twice in 3 ml ice-cold trichloroacetic acid.

The DNA and RNA precipitates were dissolved in 150 μl of 50 mM KCl and the radioactivity was determined in Luma-Gel scintillation fluid (M & B, Loughborough U.K.) using a Packard Tri-carb 2000 CA scintillation analyzer. A portion was assayed for DNA by the method of LePecq and Paoletti [14] and RNA was determined by the absorption at 260 nm. The protein was dissolved in 150 l of 0.01 M NaOH and the concentration determined by the method of Lowry *et al.* [15]. The solution was neutralised with 1 M HCl prior to radioactivity detection. All solutions of macromolecules were solubilized with hyamine hydroxide prior to counting. To establish that the agents were covalently attached to the macromolecules portions of the drug-treated DNA, RNA and protein after incubation with 0.1 mM drug for 2 hr at 37° were precipitated and washed as above and redissolved in 50 mM KCl (DNA and RNA) or 0.01 M NaOH protein. A portion of each sample was then dialysed against water for 24 hr at 4°. The undialysed and dialysed samples were then reprecipitated, washed and the macromolecular bound radioactivity was determined. After dialysis the percentage of radioactivity remaining bound to DNA, RNA and protein was for mitozolomide, 130, 99 and 88, for CCRG 81045 93, 108 and 120 and for CCRG 82019 97, 94 and 98 respectively. This suggests that all of the macromolecular bound radioactivity is covalently bound drug.

Extent of reaction of imidazotetrazinones with macromolecules in intact cells. Both Raji and GM892A cells were routinely grown in RPMI 1640 medium containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air. For incorporation experiments cells (8×10^6 ml^{-1}) were treated with sodium formate to give a final concentration of 20 mM 30 min prior to the addition of 0.1 mM (final concentration) of 3-^{14}C side-chain labelled imidazotetrazinones at the specific activities indicated in the materials section. At time intervals a portion of the cell suspension was removed, sedimented by centrifugation and washed three times in 1 ml of phosphate-buffered saline. The cell pellet was lysed in 0.5 ml of ice-cold 0.2 M perchloric acid and sedimented by centrifugation at 4°. The precipitate was washed twice with 0.5 ml of ice-cold 0.2 M perchloric acid at 4° and the washings plus the original supernatant were used to measure the acid-soluble pool. The radioactivity in the RNA in the cell pellet was determined by hydrolysis in 0.7 ml of 0.3 M KOH for 1 hr at 37° followed by precipitation with 0.5 ml of 1 M perchloric acid at 4°. The precipitate was collected by centrifugation and washed twice with 0.2 M perchloric acid. The supernatant and washings were neutralised and the radioactivity determined in Luma-Gel scintillation fluid.

The radioactivity in DNA in the pellet from the RNA determination was assayed by hydrolysis (twice) in 0.75 ml of 0.5 M perchloric acid for 30 min at 80°. On cooling the protein precipitated and the supernatant when neutralised was used for the determination of radioactivity in DNA. The remaining acid-insoluble fraction was dissolved in 1 ml of 0.01 M NaOH and the radioactivity determined after addition of 0.2 ml of hyamine hydroxide. The concentration of DNA was determined by the reaction with diphenylalanine and RNA by reaction with orcinol according to the method of Munro and Fleck [16]. To determine the efficiency of extraction of macromolecules and the extent of contamination of the various macromolecules with one another both GM892A and Raji cells were incubated with 1 μCi/ml of either (methyl-^{3}H) thymidine, (sp. act. 5.0 Ci/mmol) [5-^{3}H]uridine (sp. act. 29 Ci/mmol) or L-[4,5-^{3}H] leucine (sp. act. 67 Ci/mmol) and the DNA, RNA and protein were extracted as above. In both cell lines 96% of the (methyl-^{3}H) thymidine was associated with the DNA fraction, 97% of the [5-^{3}H] uridine was associated with the RNA fraction and 94% of the ^{14}C leucine was associated with the protein fraction.

Transport studies. These were performed essentially as described previously [17]. Cells were resuspended in fresh RPMI 1640 medium at a concentration of 5×10^6 cells ml^{-1}, and equilibrated for 10 min at 4° in an ice-water bath. Uptake was initiated by the addition of drug (0.1 mM, without dilution from the initial specific activity) in DMSO. At specified time points after drug addition, samples (200 μl) were removed to an Eppendorf tube which contained 100 ml of a silicon oil: corn oil (10:3) mixture and 50 μl of 90% formic acid. After centrifugation (9000 *g*) for 1 min the tube was frozen in liquid nitrogen, cut at the oil/acid boundary and the radioactivity was determined in Luma-Gel scintillation fluid. To establish the volume of the cells a 1 ml

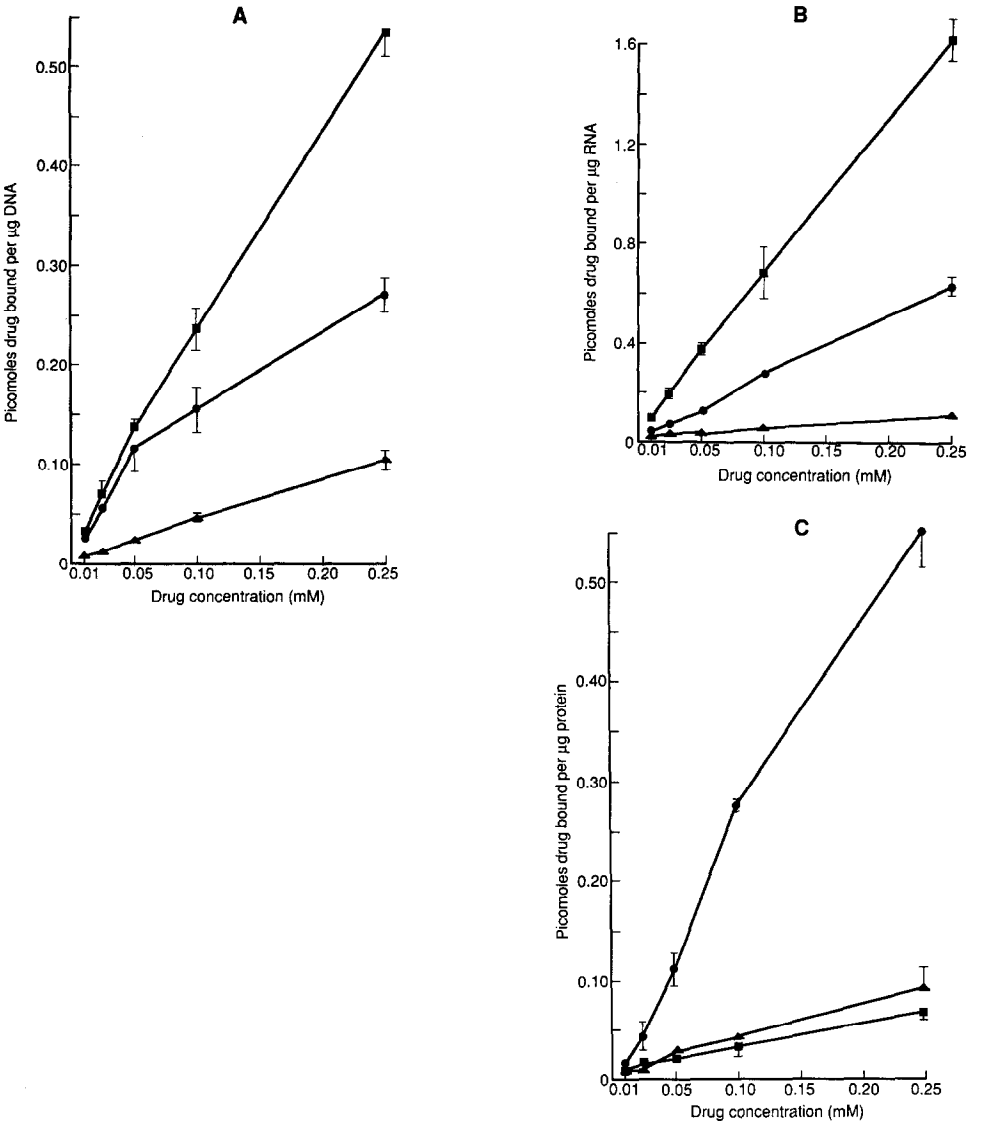


Fig. 2. Reaction of calf thumus DNA (A), calf liver RNA (B) and bovine serum albumin (C) with mitozolomide (●), CCRG 81045 (■) and CCRG 82019 (▲). Macromolecules were incubated with drugs for 2 hr at 37° and the amount of radioactivity bound was determined as described in Methods.

sample of cell suspension was treated with a mixture of ^{14}C insulin and [^{3}H]-H_2O. Once equilibrium had been established the samples were treated as above.

RESULTS

The extent of total alkylation of calf thymus DNA, calf liver RNA and bovine serum albumin by mitozolomide, CCRG 81045 and CCRG 82019 is shown in Fig. 2. The extent of reaction of all agents with all three macromolecules increases as the concentration of drug increases. For both DNA and RNA (Fig. 2 A,B) the total alkylation increases in the series CCRG 82019, mitozolomide, CCRG 81045, with CCRG 81045 being about twice as reactive as mitozolomide. However, mitozolomide is about 5–10-fold more reactive towards protein than the other two imidazotetrazinones. The alkylation of RNA exceeds that of DNA with both mitozolomide and CCRG 81045, while CCRG 82019 displays a similar low reactivity towards DNA, RNA and protein. Towards DNA, CCRG 81045 shows a five-fold greater alkylation than CCRG 82019.

To further investigate the extent of reaction of the imidazotetrazinones with macromolecules two cell lines (Raji and GM892A) were chosen with different sensitivities towards these agents (Table 1). Thus GM892A cells which have very low levels of the O^6-methylguanine methyltransferase repair protein [8] are 10- and 20-fold more sensitive to mitozolomide and CCRG 81045 than Raji cells. In contrast the ethyl analogue, CCRG 82019, shows little difference in toxicities towards the two cell lines, and in GM892A cells is 20-fold less potent than CCRG 81045 and 100-fold less potent than mitozolomide.

Table 1. Sensitivity of cell lines to imidazotetrazinones

Cell line	ID_{50} μM* (± SEM) Mitozolomide	CCRG 81045	CCRG 82019
Raji	20 ± 7	206 ± 20	360 ± 30
GM892A	2 ± 0.5	10 ± 7	229 ± 20

*Concentration required to give 50% inhibition of cell growth. Cells were plated at an initial density of 8×10^4 ml^{-1} and growth inhibition was calculated from the linear part of the growth curves. Drugs were dissolved in DMSO at 10^3 times their required concentration such that the final concentration of DMSO in the culture medium did not exceed 0.1%. Clonogenic assays on either cell line were unsuccessful.

Table 2. Initial rates of drug accumulation into Raji and GM892A cells

Cell line	Initial velocity pmol/10^6 cells/min* Mitozolomide	CCRG 81045	CCRG 82019
Raji	111 ± 4	62 ± 9.2	108 ± 7
GM892	32 ± 8	22 ± 2	66 ± 2

*Results are expressed as the average ± SEM of 3 experiments.

Differences in drug sensitivity may result from differential uptake of these agents into the two cell lines. The rate of drug uptake and the equilibrium values attained in the two cell lines is shown in Fig. 3 (A,B). As shown previously for the uptake of mitozolomide into TLX5 lymphoma cells [17] the initial rate of drug accumulation at 37° is very rapid with steady-state levels reached within 1 min. Therefore, to compare more accurately differential uptake cells were incubated at 4°, in which case equilibrium values were not attained until 4–8 min after drug addition. For all three agents the initial uptake rate is approximately linear over the first 2–4 min. The values for the initial rates of uptake into the two cell lines are shown in Table 2, and indicate that for each drug the initial uptake into Raji cells is 2–4-fold greater than into GM892A cells. The cell/medium distribution ratio remains constant for 30 min at approximately 1.3 in Raji cells and 1.8 in GM892A cells.

For all three drugs intracellular radioactivity becomes associated with macromolecules. The amount of radioactivity remaining bound to DNA, RNA and protein in the two cell lines at various times after drug treatment is shown in Fig. 4 (A,B,C). Incorporation of radioactivity via the "1-carbon pool" has been suppressed by the addition of 20 mM sodium formate prior to the addition of the labelled drug. In both cell lines the number of alkyl groups remaining bound to DNA, RNA and protein after treatment with mitozolomide exceeds that of the other two imidazotetrazinones by a factor of 4–7-fold. The extent of alkylation of DNA by mitozolomide is higher than alkylation of RNA and protein and is greater (almost 2-fold) in GM892A cells than in Raji cells. There is no difference in the overall level of alkylation of DNA in Raji cells by either CCRG 81045 or CCRG 82019 over the 24 hr period of study. In GM892A cells the overall alkylation of DNA by CCRG 81045 exceeds that of CCRG 82019 only after 24 hr despite the difference in potency of these two agents to the two cell lines. However, in contrast to Raji cells alkylation of DNA increases over a 24 hr period, presumably due to deficiencies in repair in GM892A cells. However, the total extent of DNA alkylation by CCRG 81045 and CCRG 82019 is similar in both cell lines. When compared with isolated DNA, the reactivity of these two agents is comparable in intact cells, while mitozolomide is about three times more reactive towards DNA in intact cells than to isolated DNA.

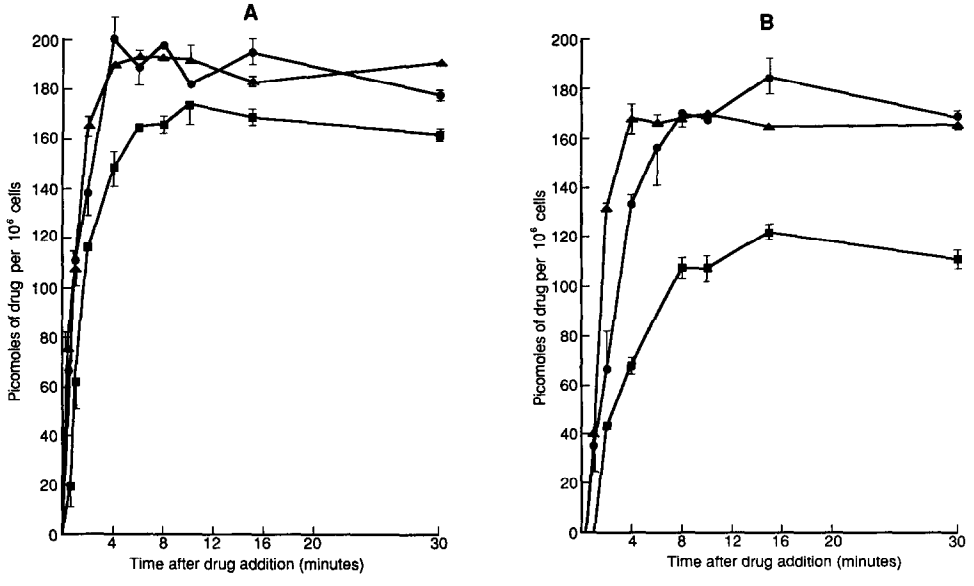


Fig. 3. Uptake of mitozolomide (●), CCRG 81045 (■) and CCRG 82019 (▲) at 4° into Raji (A) and GM892A (B) cells.

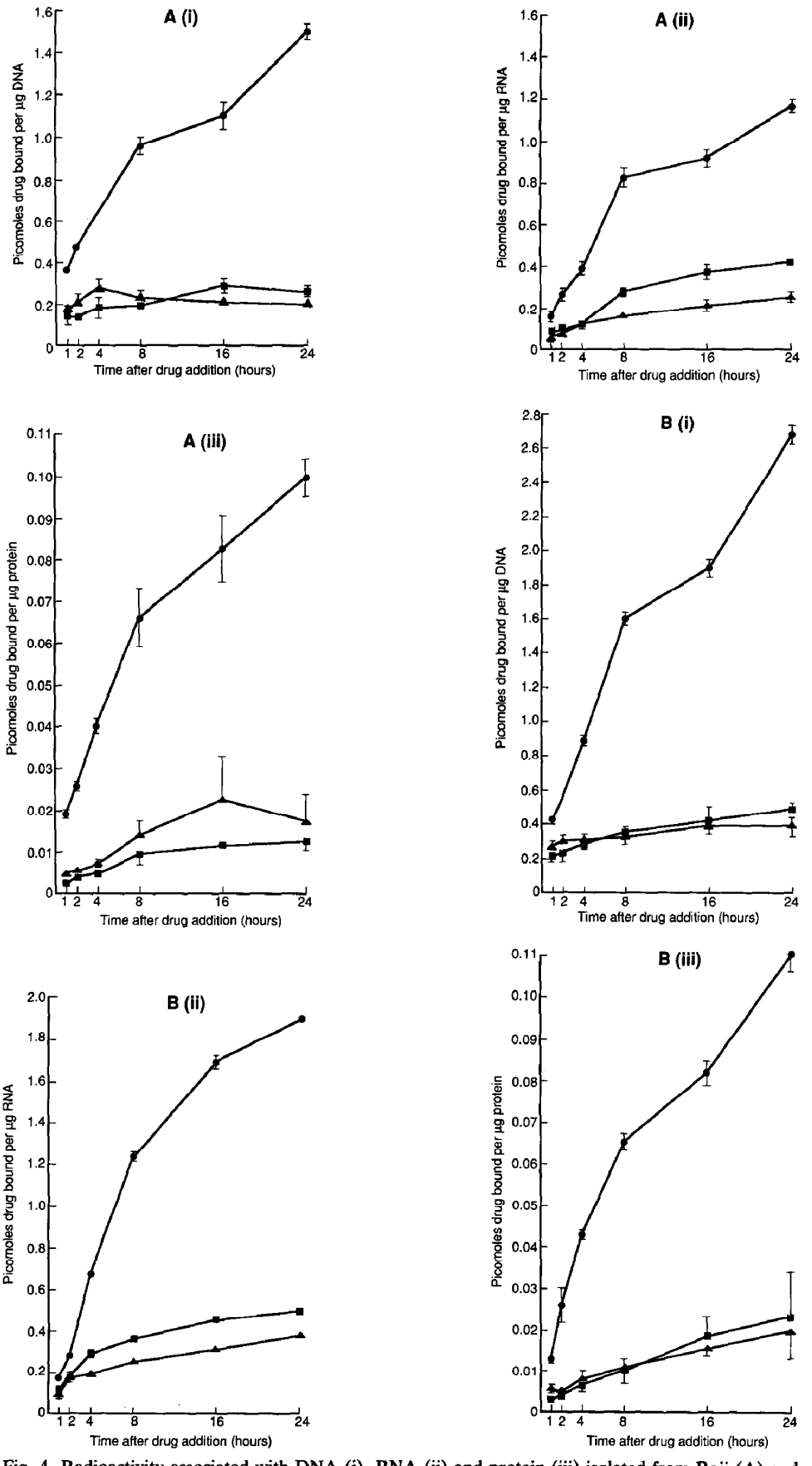


Fig. 4. Radioactivity associated with DNA (i), RNA (ii) and protein (iii) isolated from Raji (A) and GM892A (B) cells after treatment with mitozolomide (●) CCRG 81045 (■) and CCRG 82019 (▲), at stated intervals after drug addition.

The extent of reaction of both mitozolomide and CCRG 82019 with RNA in intact cells is similar to that observed with isolated RNA, while CCRG 81045 displays less alkyl groups bound to RNA in intact cells when compared with isolated RNA, presumably due to an enhanced rate of repair of this lesion. The number of alkyl groups remaining bound to RNA after treatment with mitozolomide is slightly less than to DNA, while for the other two agents binding to DNA and RNA is approximately the same.

The level of protein alkylation in intact cells is similar to that with isolated protein with all agents except mitozolomide where alkylation is about 10-fold less in cells than with bovine serum albumin. Alkylation of proteins by mitozolomide exceeds that of the other two imidazotetrazinones by a factor of about 5 and there is no difference in protein alkylation by any agent in either cell line.

DISCUSSION

The covalent binding of the antitumour imidazotetrazinones with DNA may be a critical event in the mechanism of action of these agents. Cross-resistance studies suggest a similarity in the mechanism of action of these agents to nitrosoureas and triazenes [1], agents considered to exert their cytotoxicity by reaction with specific bases in DNA. This study has attempted to determine the relative levels of reaction of 3-substituted imidazotetrazinones with DNA, RNA and protein both alone and in two cell lines which show different sensitivities to these agents. The overall level of macromolecular alkylation observed in intact cells will be a balance between the rate of alkylation and the rate of alkyl group removal by repair processes. In human fibroblasts treated with MNU 3-methyladenine is rapidly removed from DNA with a half-life of 2 hr and 7-methylguanine is removed slower with a half-life of 30 hr [18]. At low doses of MNU about 90% of the O^6-methylguanine is removed in 10 hr, but at high doses about half still remains. Also the level of 7-alkylguanine may be lower in cells treated with sodium formate to suppress incorporation via the "one-carbon pool", since it has been shown [19] that up to 30% of the total 7-methylguanine formed after treatment with dimethylnitrosamine arose by methylation via the "one-carbon pool".

Thus, whereas CCRG 81045 shows the greatest extent of alkylation of isolated DNA and RNA, in both Raji and GM892A cells mitozolomide shows the greatest extent of alkylation of all macromolecules. This difference appears to arise from a greater reactivity of mitozolomide towards DNA in intact cells, and an apparent reduced binding of CCRG 81045 to RNA in cells. Mitozolomide decomposes to form a 2-chloroethyldiazonium species [5] which is thought to be responsible for the initial alkylation and subsequent cross-linking of DNA. The rate of formation of this species is known to be dependent upon the pH of the medium and local variations in intracellular pH could account for an increased reactivity towards DNA. Alternatively chromosomal proteins may be important in directing the alkylation of DNA in cells.

While the overall level of DNA alkylation by mitozolomide would explain its enhanced cytotoxicity over the other two imidazotetrazinones there is little correlation between the alkylation of DNA in GM892A and Raji cells by CCRG 81045 and CCRG 82019 and their relative potencies. While the overall level of reaction of these agents with DNA may not be critical for cytotoxicity, alkylation at specific base positions may be more important. Thus while MNU produces predominantly 7-methylguanine as the alkylation product with DNA, ENU produces predominantly phosphotriesters [20]. However, alkylation at the O^6-position of guanine may be more important in determining cytotoxicity since the sensitivity of cell lines to these agents correlates with the level of O^6-methylguanine-DNA methyltransferase (O^6MeGMT) [8]. Also depletion of O^6MeGMT by free O^6-methylguanine sensitizes Mer^+ cells to mitozolomide and CCRG 81045, but not CCRG 82019 [8], suggesting that the ethyl analogue may produce an alternative cytotoxic lesion. Present studies are thus directed to a quantitative estimation of base alkylation by CCRG 81045 and CCRG 82019.

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