Intracellular DNA damage produced by a series of diacridines

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The intracellular DNA damage produced by a series of diacridines after a 2 h pulse treatment of L1210 cells in culture was investigated by using the alkaline-elution technique. Like other intercalating agents, diacridines produce single-strand breaks and protein–DNA links. There is a large increase in both types of damage as the alkane chain linking the two 9-aminoacridine residues is increased beyond five methylene groups, which is consistent with the previously observed change from monofunctional to bifunctional intercalation [Wakelin, Romanos, Chen, Glaubiger, Canellakis & Waring (1978) *Biochemistry* **17**, 5057–5063]. For linker chains of less than six methylene groups these agents produce less DNA damage than does the parent 9-aminoacridine at the same drug concentration. Unlike the monofunctional intercalators previously investigated [Ross, Glaubiger & Kohn (1979) *Biochim. Biophys. Acta* **562**, 41–50; Zwelling, Michaels, Erickson, Ungerleider, Nichols & Kohn (1981) *Biochemistry* **20**, 6553–6563; Zwelling, Kerrigan & Michaels (1982) *Cancer Res.* **42**, 2687–2691; Zwelling, Michaels, Kerrigan, Pommier & Kohn (1982) *Biochem. Pharmacol.* **31**, 3261–3267], there is no correlation between the number of single-strand breaks and protein–DNA links produced by these diacridines.

Bifunctional intercalating agents offer potential as pharmacologically active compounds because of their high affinity for DNA and their capacity for enhanced nucleotide sequence selectivity (Waring & Wakelin, 1974; Le Pecq *et al.*, 1975; Canellakis & Bellantone, 1976; Canellakis *et al.*, 1976*a,b*). A number of naturally occurring antitumour antibiotics have been characterized as bifunctional intercalating agents (Wakelin & Waring, 1976; Lee & Waring, 1978; Huang *et al.*, 1980) and the antitumour activity of several other classes of bis-intercalators has been investigated (Cain *et al.*, 1978; Chen *et al.*, 1978; Ohkuma *et al.*, 1980; Pelaprat *et al.*, 1980; Kuhlman & Mosher, 1981; Denny *et al.*, 1983).

The most elementary synthetic bis-intercalators are the diacridines (Fig. 1), in which two 9-aminoacridine molecules are linked via the amino group by an alkane chain. Studies using viscometric techniques have established that these compounds intercalate bifunctionally when the bridging chain reaches a critical length (Wakelin *et al.*, 1978). Thus, for linker chains of two to four methylene groups, the diacridines remain monofunctional, whereas they become bifunctional when the linker consists of six or more carbon atoms. The maximal interchromophore separation of the C₆- and C₇-diacridines is long enough for these compounds to span only a single base-pair, thereby necessitating

Abbreviations used: phosphate-buffered saline, per litre: NaCl, 8g; KCl, 0.2g; Na₂HPO₄ (anhydrous), 1.15g; KH₂PO₄, 0.2g; D-glucose, 0.2g; disodium EDTA, 0.2g (pH 7.2); Sarkosyl, sodium dodecyl sarcosinate; BSS, balanced salt solution containing, per litre: NaCl, 8g; KCl, 0.4g; D-glucose, 1.0g; Phenol Red, 2ml of a 1% solution; NaHCO₃, 0.028g; Hepes, 0.085g; gentamycin, 2 × 10⁴ units; SDS, sodium dodecyl sulphate.

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Fig. 1. Structure of diacridines
 Compounds are referred to as C_n-diacridine in the text where C_n- represents $-\text{[CH}_2\text{]}_n-$.

violation of the 'neighbour-exclusion principle' which characterizes the intercalation of monofunctional acridines and phenanthridines (Crothers, 1968; Wakelin *et al.*, 1978; Waring, 1982). The behaviour of the C₅-diacridine is enigmatic, the results obtained from viscometry measurements being atypical, and it is not clear if this compound acts as a mono- or bi-functional intercalator (Wakelin *et al.*, 1978).

The damage to intracellular DNA produced by several classes of monofunctional intercalators has been studied extensively by using the alkaline-elution technique (Ross *et al.*, 1979; Zwelling *et al.*, 1981, 1982*a,b*). All of the agents investigated produce single-strand breaks and protein-DNA links, and there appears to be a direct relationship between the lesions. These findings led to the suggestion that an enzyme causes single-strand breaks in response to the intercalation event and, further, that the enzyme attaches irreversibly to one terminus of the DNA at the break site (Zwelling *et al.*, 1982*b*). In support of this hypothesis, it has recently been shown that the intercalating antitumour agent amsacrine interferes with topoisomerase type II function by stabilizing a covalent DNA-protein intermediate (Nelson *et al.*, 1984). Our interest in the antitumour potential of bis-intercalators motivated us to investigate the DNA damage produced when mammalian cells in culture are treated with diacridines, and to ask whether this damage correlates with the results of mechanistic studies of their interaction with DNA *in vitro*. Accordingly, we have used the alkaline-elution technique (Kohn, 1978) to study DNA single-strand breaks and protein-DNA links produced by treatment of L1210 mouse leukaemia cells with these agents.

Materials and methods

Cells and cell treatment

L1210 cells in exponential-phase growth in minimal essential medium plus 15% (v/v) foetal-calf serum with added glutamine and gentamycin were treated at final drug concentrations of 5, 10, 25 and 50 μM for 2 h. Solutions of the drugs in water were prepared so that 0.1 ml added to 5 ml of cell culture gave the required concentration. Control cultures received 0.1 ml of sterile water. The drugs were removed by rapid centrifugation at 4°C, followed by resuspension of the cells in ice-cold fresh medium. Treated cell cultures were stored on ice for up to 30 min before elution and during irradiation. For radiolabelling, cell cultures were incubated with either 2-[¹⁴C]thymidine (0.02 $\mu\text{Ci/ml}$) or [methyl-³H]thymidine (0.025 $\mu\text{Ci/ml}$) for 20–24 h before drug treatment.

X-irradiation

When required, cell cultures were irradiated on ice with a Philips 250/25 X-ray generator with a 1.0 Al/0.25 Cu filter at a rate of 130 rad/min to a total of 150 or 300 rad for the single-strand break assay or 3 krad for the protein-DNA link assay.

Alkaline elution

The technique employed was similar to established procedures (Kohn, 1978). For the single-strand assays, 1 ml containing 5×10^5 ¹⁴C-labelled treated cells and 1 ml containing 5×10^5 ³H-labelled internal control cells treated with 150 rad of X-rays were suspended in 10 ml of ice-cold phosphate-buffered saline in chilled filter holders fitted with 2.0 μm -pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, CA, U.S.A.). The cells were layered on to the filter by gentle pumping (0.6 ml/min), washed with 3 ml of ice-cold phosphate-buffered saline and treated with 3 ml of lysis solution [2 M-NaCl/0.02 M-trisodium EDTA/0.3% (w/v) Sarkosyl, pH 10.0] for 30 min. This was followed by a 90 min incubation with 3 ml of lysis solution to which proteinase K had been added at a final concentration of 0.5 mg/ml. The filters were washed twice with 3 ml aliquots of 0.02 M-Na₂EDTA, pH 10.0, and the DNA was eluted with 40 ml of eluting buffer (0.09 M-tetraethylammonium hydroxide/0.02 M-H₄EDTA/0.1% SDS, pH 12.25–12.30) at a pumping rate of approx. 0.03 ml/min, 90 min fractions being collected. All treatments after cell lysis were performed in darkness or oblique light from a low-power incandescent lamp.

At the completion of the experiment the filters were carefully removed from the holders, treated with 0.4 ml of 1 M-HCl for 1 h at 60°C, then with 2.5 ml of 0.4 M-NaOH for 30 min at room temperature. Eluting buffer was used to adjust this solution to the same volume as the collected fractions, and the solution was subsequently treated in the same manner as the eluted samples. PCS scintillant (Amersham Australia Ltd.) was added to the fractions in the ratio 7.5 ml/3 ml (scintillant/fraction) and the ¹⁴C and ³H radioactivities were determined by using an LKB Rackbeta II (LKB-Wallac, Turku, Finland) liquid-scintillation counter, previously calibrated to determine d.p.m. Results were collected on an Apple II micro-computer (Apple Computer Corp., Palo Alto, CA, U.S.A.) interfaced directly to the scintillation counter for subsequent processing.

For the protein-DNA assay, experimental conditions were essentially the same, except that PVC [poly(vinyl chloride)] filters were used (Millipore Corp.); all cells received 3 krad, no proteinase K lysis solution was used and a slightly different

eluting buffer (0.112M-tetraethylammonium hydroxide/0.012M- H_4 EDTA, pH 12.25–12.3) was employed. The pH of buffers and solutions was measured with a Metrohm E632 pH-meter (Metrohm, Herisau, Switzerland) calibrated with commercially available standard buffers at pH 10 and 12.

Growth-inhibition assays

Cultures of cells in exponential-phase growth at 4×10^5 cells/ml were treated with drug at appropriate concentrations for 2h, the cells pelleted by centrifugation at 100 rev./min in a Beckman TJ-6 centrifuge (Beckman Australia) for 5 min, resuspended in BSS + 2% (v/v) foetal-calf serum, pelleted again and finally resuspended in fresh medium. Aliquots (2ml) were placed in wells in a cluster dish, incubated for 48h, and the number of cells determined using a Coulter model ZM particle counter (Coulter Electronics). Stock solutions of drug in water were prepared so that 0.1 ml added to 5ml of cell culture gave the final required concentration. Control cultures received 0.1 ml of sterile water.

Results

Growth inhibition

The activity of the diacridines against L1210 cells in culture was determined by measuring growth 48h after a 2h exposure of the cells to the drug. The concentration that produced 50% fewer cells than controls after 48h, the IC_{50} , is shown in Fig. 2 as a function of alkane chain length. A 2h exposure was also used for the DNA-damage assays. The IC_{50} values are approx. 5–10 times greater than those seen for continuous exposure of L1210 and P388 cultures to the drug (Chen *et al.*, 1978; B.C. Baguley & W. A. Denny, personal communication), but show the same trend of a large increase in potency on going from compounds that intercalate monofunctionally (C_2 – C_4) to those that bis-intercalate (C_6 – C_{12}). There is a slight decrease in the IC_{50} as the alkane chain is extended beyond C_6 (Fig. 2).

Single-strand breaks

Single-strand-break frequencies were determined by using the formula derived by Kohn (1978):

$$P = \{[\log(r_i/r_0)]/[\log(R_0/r_0)]\} P_r$$

where r_i , r_0 and R_0 are the fractions of ^{14}C -labelled DNA retained on the filter for 0rad/drug treatment, 0rad/no drug treatment and 300rad/no drug treatment respectively when 50% of the 3H -labelled internal control DNA has been eluted. P_r is the strand-break frequency due to 300 rad and P

Fig. 2. Growth inhibition of L1210 cells in culture as a function of the chain length of the diacridines (IC_{50} = concentration that inhibits growth by 50%)

is the strand-break frequency due to the drug. P_r is given the value of 300 so that the single-strand-break frequency is expressed as that produced by an equivalent dose of X-rays. The choice of 50% rather than 60% (Kohn, 1978; Ross *et al.*, 1978) is arbitrary, as it represents a common point in time at which the rate of elution is measured: the 50% point was determined by fitting the data to a cubic equation and interpolating. Theoretically the curves are linear, but we find experimentally that there is a slight curvature, so a cubic equation is chosen for accuracy of interpolation. In all cases the correlation coefficient for the curve-fitting was greater than 0.9998.

A typical elution profile of C_9 -diacridine is presented in Fig. 3(a), where for clarity the curves for only two drug concentrations are shown. The profile reveals that the DNA is eluted rapidly in the presence of the highest concentration of the drug, exceeding the rate observed for 300 rad of X-rays. Fig. 3(b) shows the elution profile for the same concentrations of C_2 -diacridine and, by contrast with the results for C_9 , only minimal elution of the DNA is found. The strand-break frequencies as a function of concentration for the diacridines with an even number of carbon atoms in the linker chain are shown in Fig. 4(a). Several features of these results merit comment. The strand-break frequencies are linearly related to dose, and this may be so even for the C_{12} -diacridine. The solubility of this homologue is such

Fig. 3. Alkaline elution single-strand-break assay for (a) C_9 - and (b) C_2 -diacridine (a) $\times$, 0 rad, no drug; $\blacktriangle$, 0 rad, $5 \mu\text{M}$ -drug; $+$, 300 rad, no drug; $\bullet$, 0 rad, $50 \mu\text{M}$ -drug. (b) $\times$, 0 rad, no drug; $\bullet$, 0 rad, $50 \mu\text{M}$ -drug; $+$, 300 rad.

Fig. 4. Single-strand-breakage frequencies for (a) even-numbered and (b) odd-numbered diacridines. The data points show the means for three experiments, the error bars indicating the range of the results. Where no error bars are shown, the error is negligible.

that, at the stock concentration required for a $50 \mu\text{M}$ solution in culture, the solution was supersaturated and precipitated on filters, pipette tips etc., so that there is an appreciable error in its concentration. There is a distinct change in the

extent of DNA damage as the linker chain length is extended from four to six methylene groups. Up to C_4 there is little increase in damage as a function of concentration, even at the higher doses, but for the C_6 and longer diacridines the strand-break fre-

quency rises rapidly with increasing drug concentration. This is also seen with the odd-numbered diacridines shown in Fig. 4(b). Again there is an approximate linear dose relationship, with the exceptions of the C₇- and C₉-diacridine, and a marked change in DNA damage produced as the homologous series traverses from mono- to bi-functional behaviour. It is interesting to note, having regard to the number of single-strand breaks produced by these agents at the same dose, that the C₅-diacridine behaves in a manner typical of the monofunctional intercalators. These findings are summarized in Fig. 5, where the single-strand-break frequency is shown as a function of alkane chain length. This Figure reveals that damage produced at each dose for the C₂- to C₅-diacridines is less than that produced by the parent 9-aminoacridine, indicated as C₀. The non-linearity in the dose-damage relationship for the C₇- and C₉-diacridines is again reflected in Fig. 5, which shows an essentially linear dependence of breakage on length of alkane chain for linkages of five methylene groups or longer at doses of 50 μ M. At lower doses this approximately linear dependence of breakage on chain length commences with the C₆-diacridine.

Fig. 5. Alkane-chain-length-dependence of single-strand-break frequencies

Data are derived from Figs. 4(a) and 4(b). Concentrations given against the curves refer to diacridines.

Protein-DNA links

The protein-DNA-link frequencies were calculated according to the 'random' model (Kohn & Ewig, 1979) by using the formula:

$$P = \{[(1 - r_i)^{-1} - (1 - r'_i)^{-1}] - [(1 - r_0)^{-1} - (1 - r'_0)^{-1}]\} P_r$$

(Laurent *et al.*, 1981), where P is the drug-induced link frequency, P_r is the strand-break frequency due to X-rays (3 krad), r_i and r_0 are the fractions of slow-eluting DNA, extrapolated to zero time, for drug-treated and untreated ¹⁴C-labelled cells respectively, and r'_i and r'_0 are the corresponding values for the fraction of slow-eluting DNA from ³H-labelled internal control cells (no drug treatment) mixed with these ¹⁴C-labelled cells. P_r is given the value of 3000 so that results are expressed in rad equivalents. The 'bound-to-one-terminus' model (Ross *et al.*, 1979) was not used as there is no reason *a priori* to assume a relationship between strand breaks and protein-DNA links, and this model gives excessively large values for the link frequency. Neither the 'random' model nor the 'bound-to-one-terminus' model yields values of the protein-DNA link frequency that correlate with the single-strand-break frequency.

The results of the assay for protein-DNA links suffer from greater errors than those of the single-strand-break measurements, owing to the two-component nature of the elution profile of this assay. Elution profiles for the C₂- and C₉-diacridines are shown in Fig. 6. The high dose of X-rays ensures that the bulk of the DNA is eluted rapidly, leaving a slow-to-be-eluted fraction that is protein-bound (Kohn & Ewig, 1979). This fraction is obtained by extrapolating the late portion of the curve back to zero time. Depending on the number of points chosen to define the line corresponding to the elution of this protein-bound DNA, significantly different values for the slow-to-be-eluted fraction can be obtained. Protein-DNA link frequencies for the diacridines are shown in Fig. 7 as a function of the interchromophore distance, and initially a trend similar to seen for the single-strand-break assay may be observed, i.e. damage comparable with, or less than, that produced by the parent 9-aminoacridine up to an alkane chain length of five carbon atoms, then a large increase as the chain is lengthened further, for doses of 50 μ M. However, the C₈-diacridine is anomalous, with the link frequency for a 50 μ M dose being in excess of 8 krad equivalents (a similar disproportionate increase is seen at 25 μ M), and the C₁₁- and C₁₂-diacridines are less effective at producing protein-DNA links, resulting in a parabolic dependence of the link frequency on chain length. The large uncertainty in the analysis means that

Fig. 6. Alkaline-elution protein-DNA-link assay for (a) C_9 - and (b) C_2 -diacridines

Fig. 7. Protein-DNA-link frequencies for diacridines as a function of alkane chain length
Points represent the means of two determinations.

little comment can be made on the results obtained at 5 and 10 μM , except that there are clearly greater numbers of protein-DNA links associated with the bis-intercalating agents.

Discussion

Diacridines are chemically inert in the sense that they are not free-radical generators, nor can they form covalent bonds with nucleic acids or proteins. Indeed, all the evidence speaks strongly for their reversible reaction with DNA, and they do not cause single-strand breaks *in vitro* (Wakelin *et al.*, 1978, 1979). Thus the damage caused to intracellular DNA reported here must be ascribable to a biochemical response to their intercalation. Observation of direct damage to intracellular DNA is consistent with the report that diacridines are taken up by cells and that, at least for the C_8 -diacridine, there is preferential localization within the cell nucleus (Canellakis *et al.*, 1976c). The most significant finding of the present work is that the extent of single-strand breaks in, and protein links to, intracellular DNA is much greater for these diacridines classified as bifunctional ligands (Wakelin *et al.*, 1978). Moreover, for the bis-intercalating agents there is an approximately linear relationship between single-strand-break frequency and linker chain length. Studies with isolated DNA suggest that the C_5 -diacridine is

anomalous, behaving as a monofunctional intercalator at low binding ratios, but as a bifunctional entity at higher levels of binding, possibly owing to ligand-induced conformational changes in the structure of the DNA helix (Wakelin *et al.*, 1978, 1979). Here we find that its ability to induce damage in intracellular DNA is characteristic of the well-defined monofunctional diacridines. Although the affinities of the C₂-C₅-diacridines for DNA are found to be indistinguishable from that of 9-aminoacridine (Wakelin *et al.*, 1979), it is noteworthy that these diacridines cause fewer single-strand breaks than does their parental monomer at the same dose. By contrast, the large increase in intracellular DNA damage seen for the bis-intercalating diacridines parallels their increased affinity for DNA (Wakelin *et al.*, 1979), although this property alone is unlikely to account for their enhanced activity. It seems more plausible that the bisintercalated ligand *per se* is a more powerful inducer of the enzymic mechanism(s) believed to produce single-strand breaks (Zwelling *et al.*, 1982a,b; Nelson *et al.*, 1984) than is a monofunctionally bound diacridine.

The above observations also generally extend to the capacity of the diacridines to induce protein-DNA links, with two very striking exceptions: the C₈-diacridine causes an extraordinarily large number of protein-DNA links, whereas the link frequency for the C₁₂-diacridine is severely diminished compared with the other bis-intercalators. The maximal interchromophore distance for C₈-diacridine is sufficiently long for the ligand to form a 'two-base-pair sandwich' in its bis-intercalated complex, a binding mode not available to the C₆- and C₇-homologues, for they are too short, and it may be this property that is responsible for the enhancement in protein-DNA links. However, since the C₉-, C₁₀-, and C₁₁-derivatives also share the capacity to form 'two-base-pair-sandwich' complexes, there must remain something unique about the conformation of the C₈-diacridine-DNA complex that promotes efficient protein-DNA linking. Measurements of the increase in contour length of DNA, as well as of helix unwinding and twist angles, do not reveal any special features for the C₈-diacridine-DNA complex compared with other bis-intercalated diacridines (Wakelin *et al.*, 1978). This suggests that the enhanced activity of C₈-diacridine may be a consequence of the formation of a ternary complex between diacridine, DNA and linking protein, in which, for example, the protein specifically interacts with the octamethylene chain of the ligand before formation of the covalent bond. The diminution of the protein-DNA-link frequency for the C₁₂-diacridine may be related to the fact that this compound is sufficiently large to span three

base-pairs in its bis-intercalated complex. Although such a large complex appears to be no impediment to the induction of single-strand breaks, it is conceivable that it may inhibit covalent attachment of the DNA-linked protein.

There is no clear relationship between the number of single-strand breaks, protein-DNA links and potency in inhibiting cellular growth among the diacridines, save the broad division that the bifunctional agents are more active in all three areas than the monofunctional ligands. The lack of correlation between the number of single-strand breaks and the number of protein-DNA links is atypical for intercalating agents. For monofunctional ligands, such as amsacrine, there appears to be a 1:1 correspondence between these parameters, provided the data are analysed by using the 'bound-to-one-terminus' model (Ross *et al.*, 1979; Zwelling *et al.*, 1981, 1982a,b). With the exception of the C₁₂-homologue, the number of protein-DNA links induced by the bifunctional diacridines is always greater than the number of single-strand breaks. This finding is difficult to reconcile simply with the involvement of a topoisomerase activity, since it is not clear how an excess of protein-DNA links could be accounted for within this model. Moreover, the difference in the dependence of strand-break and protein-link frequencies on chain length of the bisintercalating diacridines suggests involvement of two separate events for the induction of these lesions.

The absence of a correlation between either the frequency of strand breaks or protein links and growth inhibition for the diacridines accords with similar findings for other acridine, anthracycline and ellipticine drugs (Zwelling *et al.*, 1982a,b). In this regard it should be noted that the number of lesions induced by the bis-intercalating diacridines (with the exception of protein-DNA links for the C₈-diacridine) is in the range typically found for intercalating agents (Zwelling *et al.*, 1982a,b). Given the reported lack of correlation between antitumour activity and inhibition of template function for the diacridines (Chen *et al.*, 1978), it remains unclear what contribution interaction with DNA makes to their cytotoxic activity.

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