Quenching of DNA–Ethidium Fluorescence by Amsacrine and Other Antitumor Agents: A Possible Electron-Transfer Effect†

Bruce C. Baguley* and Marc Le Bret

ABSTRACT: The antitumor agent amsacrine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), when bound to double-stranded DNA, particularly poly(deoxyadenylic-thymidylic acid), reduced the fluorescence of bound ethidium without physically displacing it from DNA. Fluorescence lifetime measurements showed that the reduction of fluorescence was not due to reduction of the lifetime of the excited state of ethidium. Rather, a proportion of the DNA-bound ethidium changed to a state where the fluorescence was highly quenched. Several other 9-anilinoacridine derivatives, and also 9-hydroxyellipticine, caused quenching of ethidium–DNA fluorescence, whereas 9-aminoacridine, proflavin, and ellipticine had no effect. Resonance energy transfer (Förster transfer) is not responsible for the effect since there is no spectral overlap between the absorption spectrum of any of the agents and the fluorescence emission spectrum of ethidium. It is suggested that quenching may occur as a result of reversible formation of electron-transfer complexes between the intercalating drug and the excited state of ethidium.

Studies by Cain and co-workers in recent years have provided a large number of active experimental antitumor drugs derived from 9-anilinoacridine (Cain & Atwell, 1974; Denny et al., 1983). One derivative, amsacrine (NSC 249 992), has now proven to be clinically effective in the treatment of acute leukemia (Arlin et al., 1978). Amsacrine binds to DNA by intercalation (Waring, 1976), as does the trypanocidal drug ethidium (Waring, 1965; Le Pecq & Paoletti, 1967). In the course of determining the association constant of amsacrine to synthetic double-stranded polydeoxyribonucleotides by an ethidium displacement method, it was found that amsacrine quenched the fluorescence of ethidium when both were bound to DNA (Baguley & Falkenhaug, 1978). If the quenching of ethidium was taken into account in a separate assay, the application of the equation of McGhee & von Hippel (1974) for the competitive inhibition of ligands showing neighborhood exclusion of binding sites provided values for the association constants agreeing well with those obtained by classical methods (Baguley et al., 1981; Wilson et al., 1981).

The cause of the quenching of DNA–ethidium fluorescence was not apparent from these studies. The degree of induced quenching varied widely for different 9-anilinoacridine derivatives (Baguley et al., 1981; Baguley & Cain, 1982) and was not observed with 9-aminoacridine (Baguley & Falkenhaug, 1978). In the development of multivariate regression equations to describe the in vitro activity of a series of 48 analogues of amsacrine toward L1210 leukemia, the degree of quenching was found to be a significant variable (Baguley & Cain, 1982). Because of this possible relationship to biological activity, we have attempted to characterize more fully the mechanism of quenching of ethidium fluorescence by amsacrine.

Experimental Procedures

Agents. Amsacrine methanesulfonate and other 9-anilinoacridine derivatives were available in the Cancer Research Laboratory, Auckland, New Zealand. Ellipticine and 9-hydroxyellipticine were available in the laboratories at the Institut Gustave-Roussy, Paris, France. Ethidium bromide was a gift from the Boots Pure Drug Co., England. 9-Aminoacridine hydrochloride, proflavin, and the synthetic DNA samples were from Sigma Chemical Co.

Calculation of Binding Parameters. Equation 14 of McGhee & von Hippel (1974) was used to calculate the binding ratios of two competing agents, by using a program developed by Le Bret (1978). The DNA site size of the intercalating molecules was assumed to be 2 base pairs.

Equilibrium Dialysis. Visking dialysis tubing was treated according to the method of Stewart (1968) and used to partition two-chambered Teflon cells (each holding 0.8 mL), which were plugged with threaded Teflon plugs and shaken on an oscillatory shaker for 48 h at 25 °C. The solutions in the cells were analyzed for amsacrine and DNA content by ultraviolet absorption. The ethidium fluorescence in each cell was also measured. Total ethidium was estimated fluorometrically in the DNA-containing cell after dissociation from DNA by shaking with an equal volume of dimethyl sulfoxide–5% acetic acid (Müller et al., 1973).

Fluorescence Lifetime Measurements. Fluorescence decay times were measured by a time-correlated single photon counting apparatus constructed in the laboratory (Le Bret et al., 1977). Background noise correction and calculations of least-squares regression lines were performed with a Hewlett-Packard 9810 A calculator.

Polarization of Fluorescence. A steady-state photon counting fluorometer with a Glan polarizer and Polaroid detector filters was used. The DNA–ethidium complex was excited at 525 nm and the fluorescence measured at 595 nm. The polarization coefficient, p, was calculated by standard methods (Udenfriend, 1969; Olmsted & Kearns, 1977). If VV and VH represent the fluorescence intensities using vertically polarized light and vertically and horizontally arranged Polaroid detectors, respectively, then

$$p = \frac{VV - tVH}{VV + tVH}$$

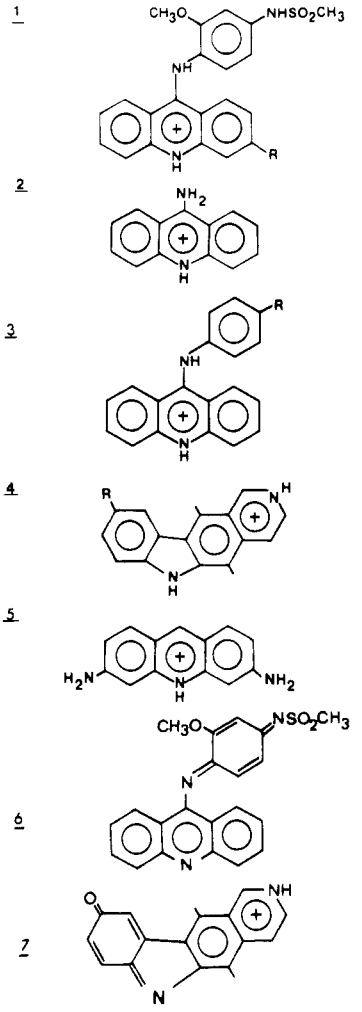
where t is a geometrical correction factor (t = HV/HH; ideally, t = 1) obtained by using horizontally polarized light.

PM2 DNA Unwinding Experiments. These were carried out as previously described (Cain et al., 1978), by using bacteriophage PM2 DNA (236 μM in phosphate) kindly

† From the Cancer Research Laboratory, University of Auckland Medical School, University of Auckland, Auckland, New Zealand (B.C.B.), and the Laboratoire de Pharmacologie Moleculaire, Institut Gustave-Roussy, 94800 Villejuif, France (M.L.B.). *Received June 22, 1983.* This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Auckland Division of the Cancer Society of New Zealand, and the Medical Research Council of New Zealand.

0006-2960/84/0423-0937$01.50/0

Chart I: Structures of Compounds Referred to in the Text



provided by Professor A. R. Morgan, Department of Biochemistry, University of Alberta, Canada. Since the 3-nitro derivative of amsacrine was poorly soluble in water, both it and ethidium were added from concentrated solutions (1 mM) in 50% aqueous ethanol. Reduced viscosities were calculated with appropriate correction for the effect of addition of corresponding volumes of 50% ethanol to the viscosity of the 9.4 mM NaCl, 2 mM Na^+HEPES, pH 7, and 20 μM EDTA buffer (0.01 SHE buffer).[1]

Results

Effect of Amsacrine on Ethidium–Poly(dA-dT) Fluorescence. Amsacrine (**1**, R = H; Chart I) decreased ethidium fluorescence when added to a solution containing poly(dA-dT) together with ethidium at a binding ratio of 0.1 molecule per base pair (Figure 1). Both the excitation spectrum (λ_{max} 530 nm) and the fluorescence emission spectrum (λ_{max} 595 nm) of ethidium remained unchanged in shape and position after each addition of amsacrine.

Ethidium (Le Pecq & Paoletti, 1967) and amsacrine bind to DNA with an apparent site size of two nucleotide pairs per drug molecule, and in 0.01 SHE buffer, they have association constants for poly(dA-dT) of 9.5×10^6 and 2.9×10^5 M^{-1}, respectively (Baguley & Falkenhaug, 1978; Wilson et al., 1981). Application of the theory of McGhee & von Hippel

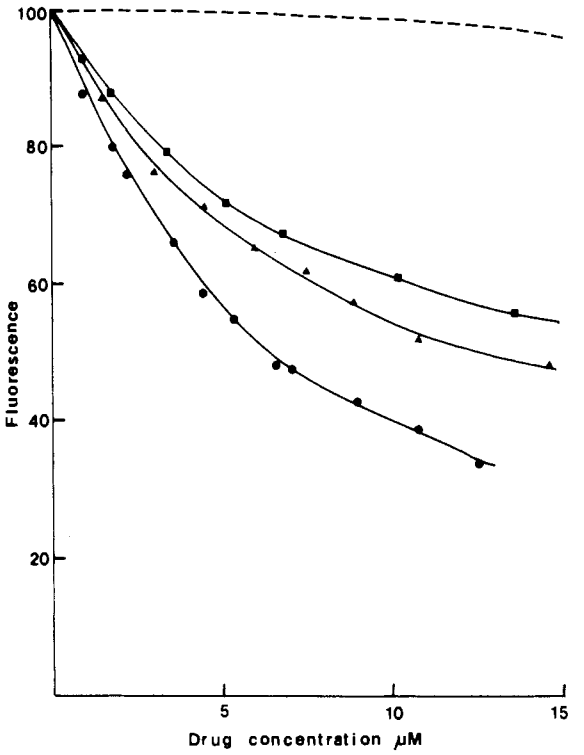


FIGURE 1: Reduction of the fluorescence intensity of ethidium–DNA complexes by addition of amsacrine. The DNA and ethidium were at concentrations of 20 μM (in base pairs) and 2 μM, respectively, in 0.01 SHE buffer. Results are shown for poly(dA-dT) (●), poly(dG-dC) (■), and calf thymus DNA (▲). The dashed line indicates the displacement from poly(dA-dT) expected on the basis of known binding data for ethidium and amsacrine. Fluorescence was monitored at 595 nm by using an excitation wavelength of 525 nm.

(1974) for competing ligands enabled theoretical ethidium displacement curves to be constructed. The curve obtained by using the above binding parameters, shown by the dashed line in Figure 1, was quite different from the observed decrease in fluorescence; a curve matching the experimental data could be obtained only by assuming unrealistically high values for the binding constant and DNA site size of amsacrine. A similar discrepancy between theoretical displacement curves and observed decreases in fluorescence was found with a range of poly(dA-dT) concentrations (2.5–80 μM in base pairs, initial ethidium binding ratio = 0.1) and a range of ethidium:poly(dA-dT) binding ratios [using 20 μM poly(dA-dT)].

To ensure that the poly(dA-dT) was in a true double-stranded form, it was first heated to 100 °C in 2 M NaCl and then dialyzed against 0.01 SHE buffer. This renatured material gave the same result as did the original sample. Fluorescence measurements were also made in 0.2 M ionic strength buffer (0.2 SHE buffer). A 20% reduction in fluorescence occurred in the presence of 3 μM amsacrine [using 20 μM poly(dA-dT)] as compared to 1.8 μM at 0.01 M ionic strength. Again, agreement with a competitive binding model was only possible if an unrealistically large DNA site size was assumed for amsacrine.

Effect of Using Different Double-Stranded Polynucleotides. Calf thymus DNA, used under conditions identical with those used for poly(dA-dT), showed the same phenomenon (Figure 1). The magnitude of the decrease was less than that for poly(dA-dT) but was virtually identical with that for high molecular weight calf thymus DNA, sheared calf thymus DNA, intact DNA from bacteriophage T7 (kindly provided by Dr. H. Jacquemin-Sablon), nicked phage PM2 DNA, and DNA derived from *Escherichia coli*, *Clostridium parvum*, and

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; 0.01 SHE, 9.4 mM NaCl, 2 mM Na^+HEPES, pH 7, and 20 μM EDTA; 0.2 SHE, same as 0.01 SHE but with 199.4 mM NaCl; poly(dA-dT), poly(deoxyadenylic-thymidylic acid).

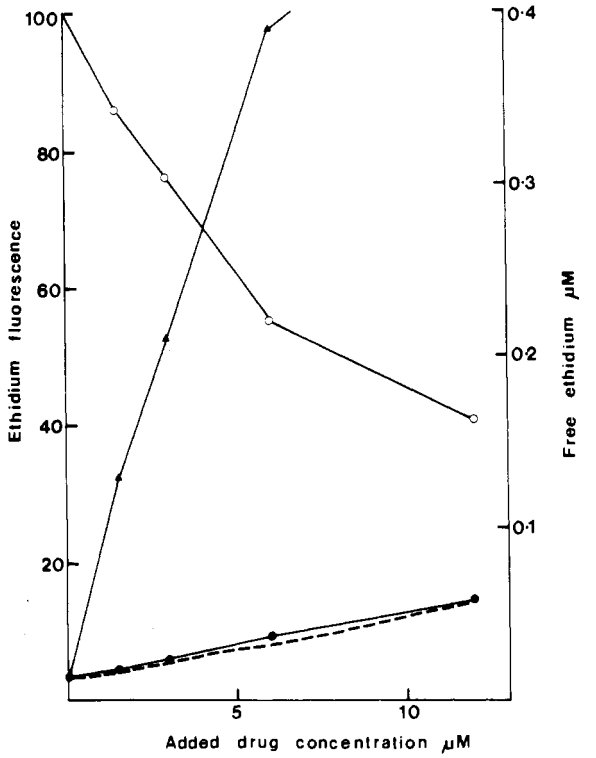


FIGURE 2: Measurement by equilibrium dialysis of the displacement of bound ethidium during the addition of amsacrine to ethidium–poly(dA-dT) complexes at 0.01 M ionic strength. The two-chambered dialysis cell contained poly(dA-dT) (20 μM in base pairs) in one side, to which was added 2 μM ethidium. For each addition of amsacrine, the fluorescence of the DNA-containing side was measured (O) (cf. Figure 1). The free ethidium concentration was measured in the DNA-free side (●). The approximate recoveries of ethidium and amsacrine were 85% and 60%, respectively (the remainder was presumably absorbed to the dialysis membrane). The dashed line indicates the concentration of free ethidium expected on the basis of known binding data for ethidium and amsacrine. If the reduction in fluorescence is equated with the liberation of free ethidium, the expected concentration of free ethidium would correspond to that indicated by the solid triangles.

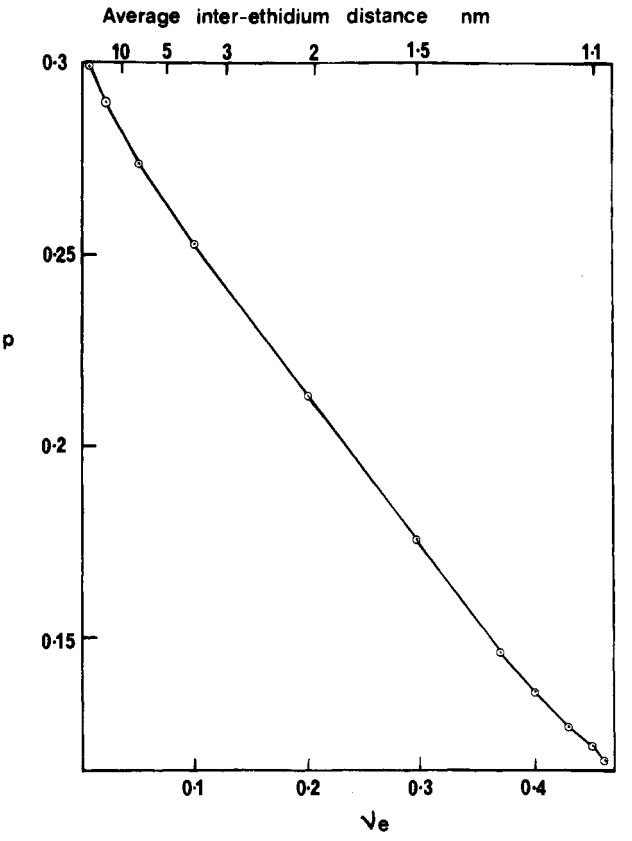


FIGURE 3: Relationship between the fluorescence polarization coefficient (p) and the ethidium binding ratio (molecules per base pair) for poly(dA-dT) (20 μM). The input ethidium concentrations were 0.2, 0.4, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, and 14 μM, from which binding ratios were calculated by using the McGhee–von Hippel equation. The average inter-ethidium distances were also calculated, by assuming that there is an interbase pair distance of 0.34 nm and that ethidium occupies a 0.34-nm slot.

Micrococcus luteus. Assuming association constants at this ionic strength for natural DNA of 2.1×10^6 M^{-1} for ethidium (Gaugain et al., 1978) and 1.8×10^5 M^{-1} for amsacrine (Wilson et al., 1981), a theoretical displacement curve could be calculated. This was very similar to that shown for poly(dA-dT) in Figure 1.

Amsacrine also reduced the fluorescence of ethidium complexes with poly(dG-dC) (Figure 1). The effect was smaller, but again was much greater than that predicted by the theoretical displacement curve.

Equilibrium Dialysis Measurements. One possible explanation for the results in Figure 1 is that amsacrine can exclude ethidium from a large DNA site (e.g., 10 base pairs). As a direct measure of ethidium displacement, amsacrine and ethidium were added to a two-compartment equilibrium dialysis cell containing poly(dA-dT) (20 μM) on one side (Figure 2). The displaced ethidium, measured fluorometrically in the DNA-free side (amsacrine is not fluorescent and does not quench the fluorescence of free ethidium), was similar to that expected by the McGhee & von Hippel (1974) model for competing intercalating molecules with site sizes of 2 base pairs. The observed decrease in the fluorescence of the complex was much greater than the observed decrease in bound ethidium.

In a further equilibrium dialysis experiment to measure the effects at higher drug binding ratios, poly(dA-dT) (20 μM) was added to one cell and either ethidium (10 μM) or amsacrine (10 μM), or both at 10 μM, was partitioned. Ethidium alone gave a binding ratio of 0.4 molecule per base pair and amsacrine alone a binding ratio of 0.25 molecule per base pair. The mixture gave binding ratios of approximately 0.38 and 0.045 per nucleotide pair for ethidium and amsacrine, respectively. (Since ethidium and amsacrine have absorption maxima at different wavelengths, direct spectrophotometric estimation of amsacrine is possible.) This result is again consistent with a neighboring site exclusion competitive binding model for both drugs.

Fluorescence Polarization Measurements. This method was used as an alternative to equilibrium dialysis to estimate ethidium displacement. When ethidium is bound at a low binding ratio to DNA, excitation by plane-polarized light leads to polarized fluorescence, since the molecule is constrained in its orientation in the time between excitation and fluorescence. The polarization coefficient under these conditions is 0.30. As the binding ratio of ethidium to DNA is increased, the polarization coefficient decreases almost linearly to a value of 0.1 (Figure 3), indicating increasing depolarization. This occurs because energy can be transferred from one bound ethidium to another, in a highly distance-dependent fashion, by resonance energy transfer (Le Bret et al., 1977). The degree of polarization of emitted fluorescent light is therefore a sensitive function of the distance between bound ethidium molecules.

In order to increase the sensitivity of the assay, the 3-nitro derivative of amsacrine (**1**, R = NO_2; Chart I), which causes more quenching than amsacrine itself (Baguley & Cain, 1982),

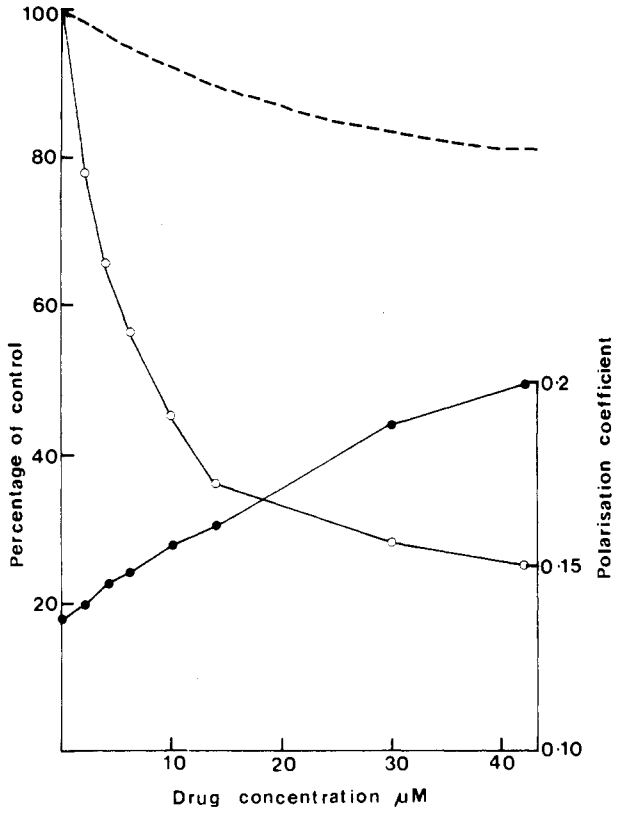


FIGURE 4: Fluorescence polarization measurements of ethidium–DNA complexes in the presence of different concentrations of the 3-nitro derivative of amsacrine. Poly(dA-dT) (20 μM in base pairs) and ethidium (0.41 bound molecule per DNA base pair, 9 μM total) were dissolved in 0.01 M SHE buffer. The fluorescence yield as a percentage of the control (○) (measured as in Figure 1) and the fluorescence polarization p (●) were measured. The displacement of ethidium was calculated on the basis of changes in the polarization coefficient (---) by using the data of Figure 4.

was used. The initial ethidium binding ratio was calculated to be 0.41 molecule per base pair. Addition of 3-nitroamsacrine caused both a decrease in the observed fluorescence and an increase in fluorescence polarization (Figure 4). The average inter-ethidium distance can be estimated by using the data from Figure 3, and with an appropriate correction for the expected lengthening of DNA caused by the intercalation of the amsacrine derivative, the ethidium binding ratio can be calculated. The calculated decrease in bound ethidium caused by the binding of 3-nitroamsacrine was markedly less than the observed decrease in ethidium fluorescence (Figure 4).

Changes in Fluorescence Lifetime. The steady-state fluorescence and fluorescence polarization measurements did not distinguish whether the fluorescence of all ethidium molecules became progressively quenched as the binding ratio of amsacrine (or its 3-nitro derivative) increased, or whether a proportion became totally quenched while the remainder was unaffected. Fluorescence lifetime measurements were therefore made by using a time-correlated photon counting technique (Le Bret et al., 1977), steady-state fluorescence measurements being carried out consecutively for each drug addition (Figure 5). Ethidium displacement was calculated to be less than 1% for added concentrations of amsacrine up to 10 μM.

Drug addition caused the fluorescence decay curve to become biphasic (Figure 5). Because of the limitations of the instrument, it was not possible to deconvolute the data and determine the magnitude of the short-lifetime component.

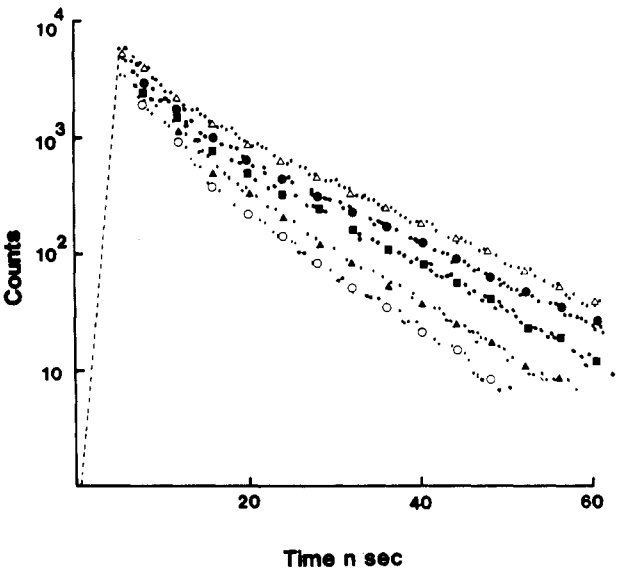


FIGURE 5: Plots of photon counts (after correction for background noise) vs. time after excitation for ethidium (8 μM)–poly(dA-dT) (80 μM in base pairs) complexes either in the absence (△) or in the presence of amsacrine at concentrations of 7.3 (●), 10.9 (■), 18.2 (▲), and 27.3 μM (○). Every eighth data point is plotted.

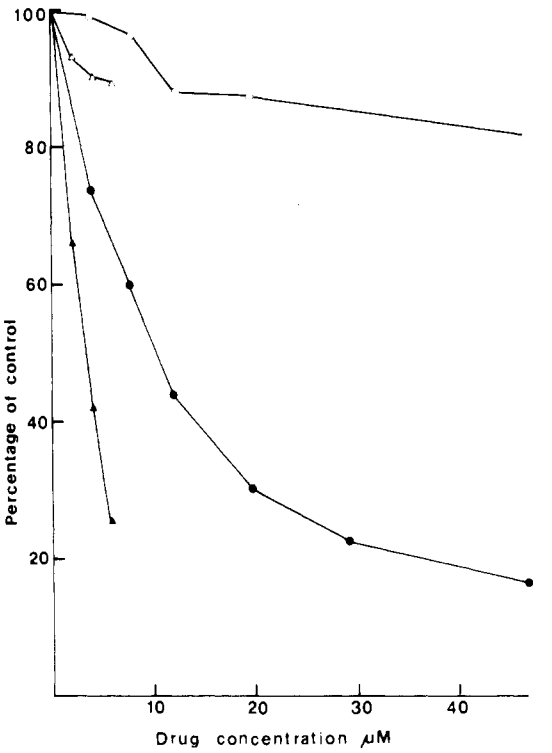


FIGURE 6: Effects of the addition of amsacrine (circles) or of its 3-nitro derivative (triangles) on the fluorescence yield and fluorescence lifetime of ethidium–DNA complexes. Fluorescence lifetimes were measured as in Figure 5, and for each addition of drug, the fluorescence intensity was measured (closed symbols) by using a steady-state photometer (cf. Figure 1). The long-lifetime component (20–60 ns) of the fluorescence lifetime was calculated by using a regression equation and expressed as a percentage of that obtained with ethidium–poly(dA-dT) alone (open symbols).

However, the long-lifetime component decreased only slightly at concentrations of amsacrine which produced large decreases in the overall fluorescence (Figure 6). Amsacrine therefore causes strong quenching of a proportion of bound ethidium molecules and has a small effect on the fluorescence lifetime of the remainder. The 3-nitro derivative of amsacrine causes even stronger quenching and a slightly greater effect on the

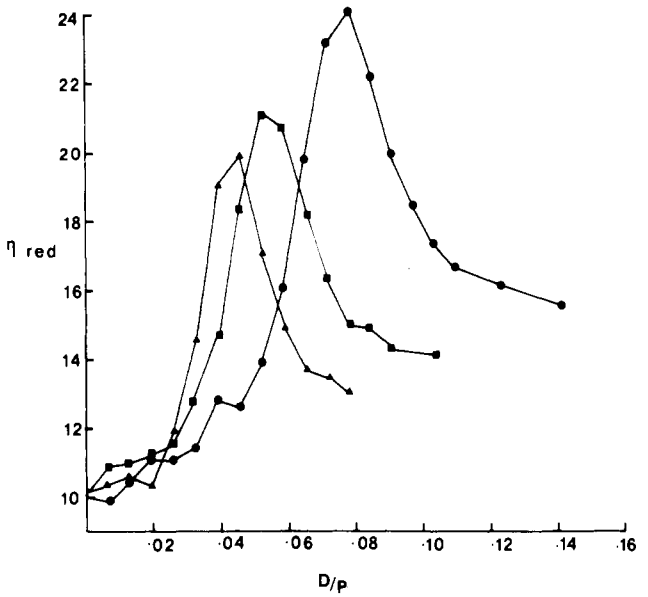


FIGURE 7: Effects of ethidium (▲), the 3-nitro derivative of amsacrine (●), and a 1:1 mixture of both drugs (■) on the viscosity of bacteriophage PM2 closed-circular DNA (236 μM in 0.01 SHE buffer). The reduced viscosity (η_{red}) at 25 °C was calculated for each added drug:DNA phosphate (D/P) ratio.

long-lifetime component (Figure 6).

Effects of Closed Circular DNA. It is possible that amsacrine in combination with ethidium could cause a major conformational change in the DNA, which leads in some way to quenching of ethidium by solvent molecules. Experiments utilizing closed circular DNA and the 3-nitro derivative of amsacrine are shown in Figure 7. The amsacrine derivative showed an apparent unwinding angle of 15.5°, assuming that of ethidium to be 26°. The stepwise addition of an equimolar mixture of 3-nitroamsacrine and ethidium provided an apparent unwinding angle of 19.3° very close to that predicted if each drug intercalates independently.

Effects of Other Intercalating Drugs. In contrast to amsacrine and its 3-nitro derivative, 9-aminoacridine and proflavin caused no decrease in the fluoresence of DNA-bound ethidium at low binding ratios. Table I compares the decrease in fluorescence observed by using the poly(dA-dT) and ethidium concentrations of Figure 1 and with a number of compounds each at a concentration of 2 μM. Under these conditions, displacement of ethidium would be expected to be less than 0.4%.

9-Anilinoacridine showed a small but significant degree of quenching; this was increased by substitution with amino groups and substituted amino groups (Table I; Baguley et al., 1981) and, in general, was not enhanced by electron-withdrawing groups. Since the derivative with a methanesulfonamide substituent (3, R = $NHSO_2CH_3$) quenches less than amsacrine, it is apparent that the electron-donating methoxy group of amsacrine also enhances the degree of observed quenching (Table I). As in the case of amsacrine (Figure 1), the use of poly(dG-dC) instead of poly(dA-dT) reduced the quenching effect by about 50% (Baguley et al., 1981).

For the series of substituted 9-anilinoacridine derivatives described by Baguley & Nash (1981), in vitro activity as measured by using cultured mouse leukemia L1210 cells is weakly but significantly correlated with the degree of quenching in this assay ($r = 0.57$; $p < 0.001$). However, since both the degrees of quenching and the DNA association constants for this series are correlated with the electron-donating capacity of the anilino substituent (Baguley et al., 1981), it is not clear whether quenching or DNA binding (or both) is important for activity. In the series of acridine-substituted derivatives of amsacrine studied by Baguley & Cain (1982), both quenching and DNA binding were independently correlated with biological activity.

Table I: Quenching of Ethidium–DNA Fluorescence by Various DNA Intercalating Agents

compd (see Chart I)	% quenching at 2 μM[a]	antitumor activity
amsacrine (1, R = H)	22	++
3-nitroamsacrine (1, R = NO_2)	45	++
9-aminoacridine (2)	0	–
proflavin (5)	0	–
anilinoacridine (3, R = H)	3	–
3, R = NH_2	41	+
3, R = $N(CH_3)_2$	49	+
3, R = OH	8	+
3, R = $NHSO_2CH_3$	19	++
3, R = Cl	3.5	–
ellipticine (4, R = H)	0	+
9-hydroxyellipticine (4, R = OH)	12	++

[a] Determinations were carried out at 0.01 M ionic strength in the presence of 2 μM ethidium and 20 μM poly(dA-dT).

Ellipticine does not cause quenching of DNA-bound ethidium, although the 9-hydroxy derivative does (Table I). Both are active antitumor agents in vivo, although the 9-hydroxy derivative is almost 100-fold more active toward L1210 cells in vitro. It is very likely that ellipticine is metabolized to the 9-hydroxy derivative in vivo to provide the active cytotoxic species (Paoletti et al., 1978).

Discussion

Certain DNA-binding agents, including the clinical antitumor drug amsacrine, have been found to reduce the fluorescence of DNA-bound ethidium to a greater extent than that expected on the basis of competition binding theory. Equilibrium dialysis and fluorescence polarization measurements indicate that this decrease is not a result solely of displacement of ethidium from DNA. The decrease, which is dependent on the type of DNA used, must therefore result from some form of quenching. Fluorescence lifetime measurements indicate that the fluorescence of a proportion of bound ethidium molecules is highly quenched, with the proportion varying for different amsacrine binding ratios. Three possible mechanisms for the quenching of ethidium fluorescence are discussed below.

(1) Förster Energy Transfer. In the presence of DNA, resonance energy transfer can occur between DNA-bound molecules of ethidium and the *p*-nitrophenyl analogue of ethidium (Le Bret et al., 1977). Energy absorbed by an ethidium molecule can be transferred by exciton transfer to the nitro analogue. The latter molecule is nonfluorescent, decaying to the ground state by a vibrational cascade. The frequency of Förster transfer varies according to the inverse sixth power of the distance between the molecules, the degree of overlap between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, and a geometrical factor (Förster, 1959; Udenfriend, 1962). The quenching of fluorescence is paralleled by a decrease in the fluorescence lifetime of ethidium, since the fluorescence yield is inversely proportional to the rate constant for the exponential decay.

The phenomenon observed with amsacrine cannot correspond to this process, because there is insufficient overlap

between the fluorescence emission spectrum of ethidium (maximum at 595 nm, range 525–650 nm) and the absorption spectrum of DNA-bound amsacrine (maximum at 442 nm). Approximately 1% of the amsacrine absorption spectrum overlaps with the ethidium fluorescence spectrum. The same argument applies to the other 9-anilinoacridine derivatives and to the ellipticine derivatives, which have absorption maxima in the same range (425–460 nm) as that of amsacrine. In contrast, the nonfluorescent nitro derivative of ethidium, which is thought to quench ethidium fluorescence by Förster transfer (Le Bret et al., 1977), has an absorption maximum at 540 nm, providing considerable spectral overlap.

The fluorescence lifetime measurements also argue against Förster transfer, since the decrease in fluorescence lifetime (Figures 5 and 6) is not paralleled by the decrease in fluorescence yield. However, this argument alone is not conclusive, since if the distribution of ethidium and amsacrine molecules is nonrandom, the fluorescence lifetime of some ethidium molecules may be affected more than others (Le Bret et al., 1977). It is probable that the small decrease in the long-lifetime component observed for amsacrine and the nitro derivative (Figure 6) is a result of resonance energy transfer between quenched and unquenched ethidium molecules, an analogy with the results of Le Bret et al. (1977).

(*2*) *Solvent-Induced Quenching.* Olmsted & Kearns (1977) have suggested that in the presence of a solvent (particularly water), an ethidium molecule, following elevation to an excited state (EH^*), can lose a proton from one of its amino groups. The resulting neutral species decays back to the ground state by a vibrational cascade and then reaccepts a proton from the solvent. This nonradiative process causes ethidium to have a fluorescence lifetime of 1.8 ns in aqueous solution, whereas if it is immobilized in various frozen salt solutions at 77 K, or intercalated between the base pairs of DNA, its fluorescence lifetime is much longer (18–28 ns, depending on the environment).

$$\begin{array}{ccc} EH^{+*} + S & \rightleftharpoons & E^* + SH^+ \\ \uparrow h\nu & & \downarrow \\ EH^+ + S & \rightleftharpoons & E + SH^+ \end{array}$$

If solvent-induced quenching is responsible for the observed effect, it would have to be assumed that the binding of amsacrine to DNA induces a conformational change such that DNA-bound ethidium at a more distant site is able to interact to a greater extent with water molecules. In particular, if ethidium is displaced from an intercalated to an outside binding mode, such a change in solvation would be possible. However, it is clear from the equilibrium dialysis data, particularly when both ethidium and amsacrine are present at concentrations of 10 μM, that the binding constant of ethidium is not markedly changed in the presence of amsacrine. A change to binding at an outside site would be accompanied by a decrease in binding affinity. Furthermore, the data with closed circular DNA (Figure 7) indicate that if a major conformational change occurs in the DNA, it is not evident from the hydrodynamic properties of the DNA. Therefore, it is unlikely that amsacrine or the other compounds in Table I could induce solvent-mediated quenching of DNA-bound ethidium.

(*3*) *Photoinduced Electron Transfer.* It is well established that in polar solvents such as acetonitrile, electronic excitation of a fluorescent electron acceptor (F^*) can give rise to electron-transfer complexes ($F^*\cdots Q$) with excited or nonexcited electron donors (Q). This process generally results in fluorescence quenching and may be either reversible or irreversible (Rehm & Weller, 1970).

$$\begin{array}{ccccc} F^* + Q & \rightleftharpoons & F^*\cdots Q & \rightleftharpoons & F^{*-}\cdots Q^+ \\ \uparrow h\nu & & & & \downarrow \\ F + Q & & \rightleftharpoons & & F^-\cdots Q^+ \end{array}$$

Much work has been carried out with transition-metal complexes [e.g., see Ballardini et al. (1978)] or with polycyclic hydrocarbons (Miller et al., 1982), using aromatic amines such as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine as quenchers. The use of rigid matrices such as glasses in these studies has indicated that electron transfer can occur over distances up to 15 Å (Miller et al., 1982). Analogous studies using molecules immobilized in lipid monolayers have shown electron transfer over distances up to 40 Å (Penner & Möbius, 1982). It is therefore pertinent to ask whether electron transfer could occur along the axis of the DNA double helix. It has been known for some time that illuminated complexes of cationic dyes with nucleic acids produce electron paramagnetic signals characteristic of electron transfer (Balazs et al., 1968).

Amsacrine is a substituted phenylenediamine, and its metabolism (Shoemaker et al., 1982) involves the oxidation to the corresponding quinone imine (**6**, Chart I). Among the substituted 9-anilinoacridines, those with the greatest quenching effect are those with an amino or substituted amino group para to the acridinylamino group (Table I; Baguley et al., 1981). These compounds, by analogy with phenylenediamine, would be expected to be susceptible to oxidation to the corresponding quinone imine. Similarly, 9-hydroxyellipticine (but not ellipticine) is an analogous system potentially oxidizable to the quinone imine [**7**, Chart I; see Paoletti et al. (1978)]. This susceptibility to oxidation should parallel the ability of these compounds to act as electron donors in forming electron-transfer complexes. Thus, amsacrine and certain related compounds might be expected to function as quenchers with the appropriate fluorochrome.

Ethidium is not a strong oxidizing agent, although it does participate in photoinduced oxidation reactions (Olmsted & Kearns, 1977). Electronic excitation should decrease the ionization potential and increase the probability of electron-transfer reactions (Ballardini et al., 1978). The acquisition of an electron by one of the amino groups is electronically feasible and can be compared to the loss of a proton in the mechanism proposed for solvent-induced quenching.

We therefore propose that, in contrast to the situation in aqueous solvents where the quenching of ethidium is mediated by reversible proton transfer, DNA-bound ethidium may be quenched by reversible electron transfer. In particular, we propose that under the conditions described here, a proportion of the excited ethidium species form electron-transfer complexes with DNA-bound amsacrine. Following electron transfer, ethidium decays to the ground state by a vibrational cascade and then transfers the electron back to amsacrine. One implication of the data in Figure 1 is that the efficiency of formation of electron-transfer complexes may be dependent on the base composition of the DNA.

This model explains why 9-anilinoacridines and 9-hydroxyellipticine show the effect, whereas simple aminoacridines and ellipticine do not. Confirmation of the hypothesis will involve testing the ability of amsacrine and other compounds to function as quenchers in other electron-transfer reactions and a search for transient electron-spin resonance signals which should be produced in such complexes. The combination of ethidium and amsacrine as an electron do-

nor–acceptor system where Förster transfer is not possible because of spectral properties is a promising potential system for more detailed mechanistic studies.

In conclusion, it is important to remember that the quenching phenomenon, at least for the derivatives of 9-anilinoacridine examined so far, is related to antitumor activity. Although it is possible that the oxidation potential is important for some other reaction of the drug while it is separated from the DNA, the properties of this series of anilinoacridine drugs suggest that the majority of intracellular drug will be DNA bound (Wilson et al., 1982). Thus, the effects of electron donation into the DNA double helix, as an electron-conducting macromolecule, could provide a basis for new theories on the mode of action of DNA-binding antitumor drugs.

Acknowledgments

B.C.B. particularly thanks the Institut National de la Santé et de la Recherche Médicale and the Centre National de la Recherche Scientifique for a grant which enabled him to work 3 months at the Laboratoire de Pharmacologie Moleculaire, Villejuif, France, and also thanks Professor C. Paoletti and Dr. J.-B. Le Pecq for their help and discussions during this period.

Registry No. **1** (R = H), 51264-14-3; **1** (R = NO_2), 64895-35-8; **3** (R = H), 3340-22-5; **3** (R = NH_2), 58658-11-0; **3** [R = $N(CH_3)_2$], 13365-38-3; **3** (R = OH), 61421-83-8; **3** (R = $NHSO_2CH_3$), 53478-38-9; **3** (R = Cl), 61462-75-7; **4** (R = OH), 51131-85-2; ethidium, 3546-21-2; poly(dA-dT), 26966-61-0; poly(dG-dC), 36786-90-0.

References

Arlin, A. Z., Sklaroff, R. C., Gee, T. S., Kempin, S. J., Howard, J., Clarkson, B. D., & Young, C. W. (1980) *Cancer Res. 40*, 3304–3306.

Baguley, B. C., & Falkenhaug, E. M. (1978) *Nucleic Acids Res. 5*, 161–171.

Baguley, B. C., & Nash, R. (1981) *Eur. J. Cancer 6*, 671–679.

Baguley, B. C., & Cain, B. F. (1982) *Mol. Pharmacol. 22*, 486–492.

Baguley, B. C., Denny, W. A., Atwell, G. J., & Cain, B. F. (1981) *J. Med. Chem. 24*, 170–177.

Balazs, E. A., Young, M. D., & Phillips, G. O. (1968) *Nature (London) 219*, 154–156.

Ballardini, R., Varani, G., Indelli, M. T., Scandola, F., & Balzani, V. (1978) *J. Am. Chem. Soc. 100*, 7219–7223.

Cain, B. F., & Atwell, G. J. (1974) *Eur. J. Cancer 10*, 539–549.

Cain, B. F., Baguley, B. C., & Denny, W. A. (1978) *J. Med. Chem. 21*, 658–668.

Denny, W. A., Baguley, B. C., Cain, B. F., & Waring, M. J. (1983) *Molecular Aspects of Anticancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 1–34, Macmillan & Co. Ltd., London.

Förster, T. (1959) *Discuss. Faraday Soc. 27*, 7–17.

Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., & Le Pecq, J.-B. (1978) *Biochemistry 17*, 5078–5087.

Le Bret, M. (1978) *Biochemistry 17*, 5087–5088.

Le Bret, M., Le Pecq, J.-B., Barbet, J., & Roques, B. P. (1977) *Nucleic Acids Res. 4*, 1361–1379.

Le Pecq, J.-B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.

Le Pecq, J.-B., Dat-Xuong, N., Gosse, C., & Paoletti, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5078–5082.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.

Miller, J. R., Peeples, J. A., Schmitt, M. J., & Closs, G. L. (1982) *J. Am. Chem. Soc. 104*, 6488–6489.

Müller, W., Crothers, D. M., & Waring, M. J. (1973) *Eur. J. Biochem. 39*, 223–234.

Olmsted, J. O., III, & Kearns, D. R. (1977) *Biochemistry 16*, 3647–3654.

Paoletti, C., LeCointe, P., Lesca, P., Cros, S., Mansuy, D., & Dat-Xuong, N. (1978) *Biochimie 60*, 1003–1009.

Penner, T. L., & Möbius, D. (1982) *J. Am. Chem. Soc. 104*, 7407–7413.

Rehm, D., & Weller, A. (1970) *Isr. J. Chem. 8*, 259–271.

Shoemaker, D. D., Cysyk, R. L., Padmanabhan, S., Bhat, H. B., & Malspeis, L. (1982) *Drug Metab. Dispos. 10*, 35–39.

Stewart, C. R. (1968) *Biopolymers 6*, 1738–1743.

Udenfriend, S. (1962) *Fluorescence Assay in Biology and Medicine*, Vol. 1, Academic Press, New York and London.

Udenfriend, S. (1969) *Fluorescence Assay in Biology and Medicine*, Vol. 2, Academic Press, New York and London.

Waring, M. J. (1965) *J. Mol. Biol. 13*, 269–282.

Waring, M. J. (1976) *Eur. J. Cancer 12*, 995–1001.

Wilson, W. R., Baguley, B. C., Wakelin, L. P. G., & Waring, M. J. (1981) *Mol. Pharmacol. 20*, 404–414.