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BIOLOGICAL ACTIVITY AND MOLECULAR INTERACTION OF A NETROPSIN–ACRIDINE HYBRID LIGAND WITH CHROMATIN AND TOPOISOMERASE II

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Abstract—A hybrid molecule, which combines an anilinoacridine chromophore related to the antitumour drug amsacrine (m-AMSA) and a bispyrrole moiety analogous to the antiviral agent netropsin, has been examined for its ability to bind chromatin and to modulate the activity of topoisomerase II. The results show that the presence of histones does not alter the bimodal DNA binding process. Intercalation of the acridine and groove binding of the netropsin part of the drug are both observed with chromatin preparations. Moreover, the hybrid has a clear topoisomerase II–DNA cleavable complex-inducing activity close to that of m-AMSA. The role of the two parts of the hybrid ligand is discussed in relation to ternary complex formation. Two cell lines (L1210 leukemia and MCF7 mammary carcinoma) were compared in their sensitivity to the tested ligand. The drug, which appears to be an efficient growth inhibitor of leukemic cells *in vitro*, reveals moderate activity against P388 leukemia *in vivo*. The biological activity of the hybrid may derive from a mechanism that involves DNA binding and topoisomerase II inhibition. This study demonstrates that agents which intercalate and bind to the minor groove of DNA simultaneously represent a new class of drugs interfering with topoisomerase II and provide opportunities for the development of new antitumour agents.

The continuous spread of cancer and the large number of malignancies that do not respond to chemotherapy call for the development of safe and effective antitumour drugs. A serendipitous approach, which consists of testing any kind of synthetic chemical or natural product for ability to inhibit cancer cell proliferation, was initially launched. This irrational stochastic screening has had the merit of yielding a number of anticancer drugs with an established place in the clinic (doxorubicin for example). Another approach was to identify molecular targets that could be exploited in the rational design of selective antitumour drugs.

DNA embodies the genetic specification of an organism and directs cellular proliferation and differentiation. Accordingly, it has been considered as a potential cellular target for drug action [1, 2]. For more than a decade, extensive effort has been directed towards the synthesis and study of DNA-interacting agents, primarily based on their potential as antitumour drugs. Chemists have focused their attention primarily on the design of powerful drugs able to bind DNA with an extreme affinity.

Since the 1970s, new dimensions were added to our understanding of cell control proliferation as a result of the molecular biology of genes. This, combined with the advent of footprinting techniques [3] which have revealed the sequence-dependent nature of the binding of various antibiotics, has recently kindled productive interest in developing DNA sequence-specific drugs [4]. Over the past 5 years, there have been significant advances in our knowledge of the way in which DNA can be targeted at definite sequences either by specific groove binding [5, 6] or site-selective intercalation [7, 8].

Simultaneously, a landmark discovery was the identification of the importance of topoisomerase II (EC 5.99.1.3), an enzyme that alters the degree of supercoiling and twisting of DNA [9–11], in mediating the antitumour activity of intercalating agents such as amsacrine (m-AMSA¶) [12]. Topoisomerase II introduces a transient double-strand break in DNA and forms a protein–DNA complex referred to as the cleavable complex. Some cancer chemotherapeutic agents, consisting mainly of DNA-intercalating drugs, trap the enzyme at this stage stabilizing the cleavable complex and thus preventing the restoration of intact DNA strands [13–16].

The exact molecular basis for the interference with topoisomerase II remains unknown, but it is likely that the drug-induced site-specific structural distortions of DNA are determinant. However, no evidence was found for direct correlation between the unwinding potentials of intercalating agents and their effects on the catalytic activity of topoisomerase

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¶ Abbreviations: m-AMSA, amsacrine; ELD, electrical linear dichroism.

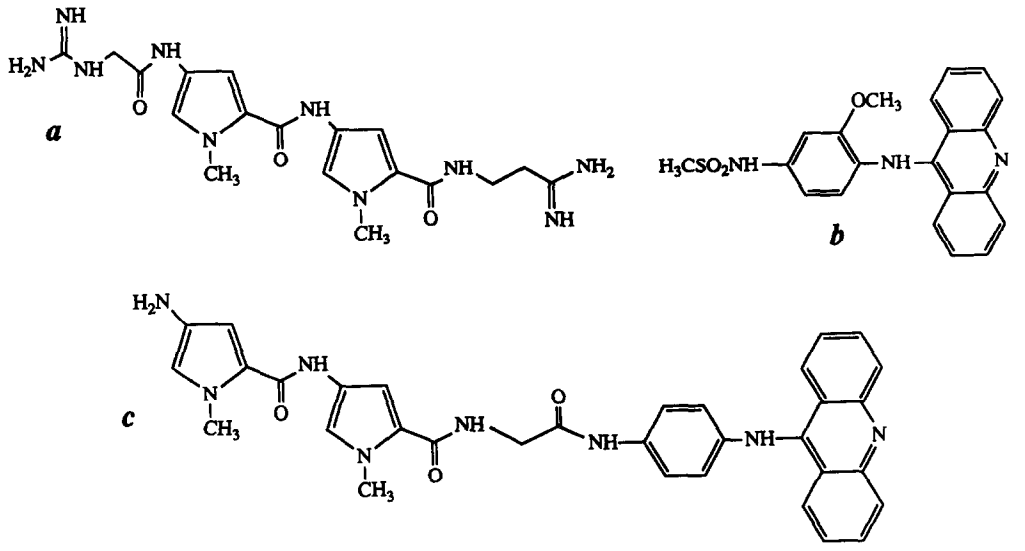

Fig. 1. Chemical structures of netropsin (a), m-AMSA (b) and the hybrid ligand NetGA (c).

II [17]. In contrast, correlations have been found between cytotoxicity and topoisomerase II-induced DNA breakage [18].

On the other hand, minor groove binders are able to impede the catalytic activity of topoisomerases I [19, 20] and II [21–23] and are now regarded as a new class of topoisomerase II-interfering agents providing new opportunities for the modulation of these proteins.

Having these different results in mind, we postulated that the combination of minor groove binders and intercalators has potential usefulness as an antitumour agent. Hybrid ligands—which we named "combilexins" [24]—were designed with the rationale that the intercalating chromophore may provide *DNA affinity* and that the appended pseudopeptide may support the *DNA sequence selectivity*. Moreover, it is also expected that these effects, based upon mere consideration of DNA as the primary target for antitumour action, may lead to the discovery of topoisomerase II-mediated selective antitumour agents. The present paper describes some aspects of the biochemical pharmacology of a model hybrid compound.

A series of minor groove binding–intercalating hybrid compounds consisting of a netropsin skeleton conjugated to an anilino-aminoacridine nucleus structurally related to m-AMSA has been synthesized [25]. In the present study, we have explored the antitumour activity of one of these hybrid compounds, NetGA (Fig. 1), for which the mechanism of binding to DNA (i.e. intercalation and groove binding) is well elucidated [26]. While the effect of this drug may be understood in terms of interaction with DNA, it is obviously necessary to know if such a bimodal binding can be observed at the chromatin level. Therefore, in parallel, we report its binding mode to chromatin and its interaction with topoisomerase II which may be a key mechanism in its antitumour activity. We found that the drug inhibits the proliferation of tumour cells *in vitro* and exhibits moderate, but noticeable, antitumour effects *in vivo*. We observed that the drug binds chromatin by two distinct processes and is as efficient as the antitumour drug m-AMSA in stabilizing the topoisomerase II–DNA cleavable complex. The results support the view that hybrid compounds are promising in terms of DNA binding, topoisomerase II inhibition and antitumour activity.

MATERIALS AND METHODS

Drugs

The synthesis of NetGA together with complete spectral characterization was described previously [25]. Fluorouracil was purchased from Laboratoires Roche (France). m-AMSA was obtained by a modification [27] of the initial procedure of Cain *et al.* [28]. Netropsin and distamycin were obtained from the Aldrich Chemical Co. (Gillingham, U.K.) and Serva (Heidelberg, F.R.G), respectively.

Preparation of chromatin fibres and electric linear dichroism

The chromatin was extracted from chicken erythrocytes and purified according to described procedures [29]. Details of the procedures used for ELD experiments were reported previously [26]. In the ionic strength conditions (cacodylate 1 mM pH 6.5) used for ELD measurements, chromatin (10–80 nucleosomes long) is mostly present as the 10 nm fibre.

DNA topoisomerase II-mediated DNA cleavage

Calf thymus DNA topoisomerase II and covalently closed circular pSP65 DNA were purified according to published procedures [30–32]. The DNA topoisomerase II cleavage reaction mixture (15 μL total volume) contained 13.2 μg/mL pSP65 DNA, 28.2 μg/mL DNA topoisomerase II, 1 mM ATP and drug at the indicated concentration in 100 mM KCl, 0.5 mM Na_3EDTA, 0.5 mM dithiothreitol, 30 μg/mL bovine

albumin and 40 mM Tris–HCl pH 8.0. After incubation at 37° for 15 min, sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.35% and 90 μg/mL, respectively, and samples were incubated further at 50° for 45 min. The digestion was stopped by addition of 5 mL of 0.05% bromophenol blue, 50 mM Na_3EDTA, 50% sucrose. DNA was subjected to electrophoresis in a 5% polyacrylamide gel at 15 V/cm for 2 hr at room temperature. After staining with ethidium bromide, the gel was photographed under UV illumination using Polaroid Film. The negative was scanned and peak area, measured with a Joyce-Loebl chromoscan 3, was converted into DNA mass using a standard 25 ng of linear pSP65 DNA obtained by digestion of the circular form with *Hind*III restriction endonuclease.

Cell cultures, growth inhibition and clonogenicity assays. L1210 leukemia cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% foetal calf serum (v:v) in a humidified atmosphere of 5% carbon dioxide in air at 37°. Fresh aliquots, stored in liquid nitrogen, of cells were thawed frequently. Cultures used to assess drug effects were in exponential growth (doubling time 15–18 hr). The drug at the appropriate concentration was added to cell cultures (5 mL) for 4 days without renewal of the medium. Every day, the cells were diluted with Trypan blue and counted. Assays were carried out in triplicate and the results averaged.

MCF7 human mammary carcinoma cells were maintained in Dulbecco MEM (GIBCO, Uxbridge, U.K.) medium supplemented with 10% foetal calf serum (v:v) in a humidified atmosphere of 5% carbon dioxide in air at 37°. To establish growth curves under continuous exposure to NetGA at various concentrations, 1.5×10^5 exponentially growing MCF7 cells were seeded in 2 mL Leighton tubes and incubated at 37° for 8 days. Every day the cells were harvested with 0.1% Trypsin and counted using a Coulter counter (D230). Four tubes were used for each point. A minimum of three separate experiments was carried out for each concentration of both drugs. Survival curves, representative of the toxicity of the tested drug, were determined by using the colony forming method [33]. Depending upon the number of surviving colony forming units expected after a given treatment, different but known numbers of cells were plated in a dish. The number of cells seeded was such that an average of 100 colonies per bottle was obtained. Cells were incubated for 15 days in 10% carbon dioxide atmosphere. These cells were exposed continuously to different concentrations of NetGA or m-AMSA without renewal of the growth medium during the experiments.

Toxicity studies in mice. These were performed using healthy female Swiss mice (median weight: 30 g, range 28–31 g). NetGA was administered i.p. in 0.4 mL of sterile physiologic saline. Three serial dosages (5, 25, 50 mg/kg/day) were tested by daily administration from day 1 to day 5. Each dosage was administered to three mice. Toxicity was evaluated both by the evolution of animal weights and by 30 days survival.

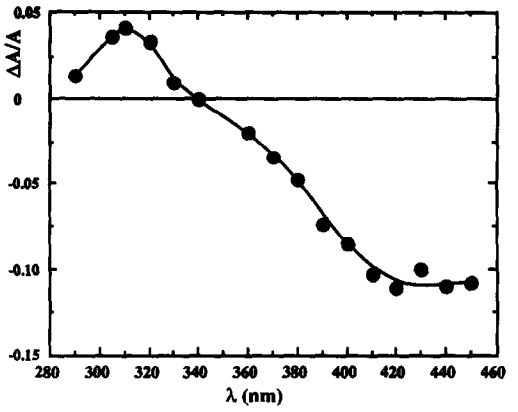

Fig. 2. Reduced linear dichroism (ΔA/A) spectra of a NetGA–chromatin complex, at a drug to DNA-phosphate ratio of 0.1 and at 12.5 kV/cm in 1 mM Tris–HCl buffer, pH 7.

Antitumour activity in mice. This was evaluated in P388-bearing DBA/2 female mice (Charles River, France) (median weight: 23.7 g, range 23.4–24.8 g). Mice were housed six per cage with free access to food and water. Inoculum consisted of 0.1 mL of diluted (sterile physiologic saline) ascitic fluid containing 10^6 leukemic cells drawn from a leukemic mouse. The day of i.p. tumour inoculation was designated as day 0. NetGA was administered i.p. (25 mg/kg/day) in 0.2 mL of sterile physiological saline from day 1 to day 5. Untreated controls and positive controls were performed by i.p. administration of sterile physiological saline (0.2 mL, day 1 to day 5) and 5-fluorouracil (20 mg/kg/day in 0.4 mL sterile physiological saline, day 1 to day 5), respectively. Median and range of survival time were reported for each group. Antitumour activity was evaluated by calculating T/C% = (test group median survival time/control median) × 100. Comparison between groups were performed using the non-parametric Mann-Whitney U-test.

RESULTS

Mode of binding to chromatin fibres

A previous investigation revealed that the drug fits isohelically in the minor groove of DNA by its netropsin moiety and intercalates its acridine chromophore between DNA base pairs [26]. In order to determine the influence of proteins bound to DNA on the binding characteristics of NetGA, its interaction with chicken erythrocyte chromatin was studied using the electric linear dichroism technique which can independently reveal the orientation of both the netropsin and the acridine moieties of the hybrid. The ELD signal depends on the local orientation of the light-absorbing chromophores relative to the orientation axis of the chromatin fibres. Upon binding to chromatin fibres, the ligand displays two bands of opposite signs (Fig. 2). The positive signal at 310 nm reflects the position of the bispyrrole unit while the negative signal centred at

430 nm reflects the position of the acridine moiety. Assuming, by virtue of electrodynamic arguments [34], that the chromatin fibres are parallel to the electric field, the negative sign of the dichroism in the 430 nm band of the hybrid ligand and the band of the DNA at 260 nm suggest an intercalation of the acridine moiety. Opposingly, the positive dichroism at 310 nm supports a groove binding process for the netropsin moiety.

The field strength dependence of the reduced dichroism of a chromatin–NetGA complex is unequivocal in that the dichroism amplitude of the complex at 430 nm is a little higher than that of chromatin alone monitored at 260 nm (not shown): at 12.5 kV/cm, reduced dichroism values of approximately −0.08 and −0.12 are observed at 260 and 430 nm, respectively. In the 310 nm region, both NetGA and netropsin exhibit positive dichroism signals, supporting the view that netropsin and the netropsin-like moiety of the hybrid NetGA adopt a similar configuration upon binding to chromatin. Thus the mode of interaction of NetGA with chromatin remains qualitatively similar to that observed with naked DNA, with minor modifications in its binding affinity. Upon interaction with DNA, the absorption spectra of the ligand showed a large red shift and 27% hypochromism. In the presence of chromatin, the hypochromic effect increases to reach 47%. The knowledge of the binding mode of this drug may be a crucial element for the interpretation of the mode of interaction with the topoisomerase II–DNA complex.

Stabilization of the topoisomerase II–DNA complex

m-AMSA induces large amounts of protein-associated DNA breaks in cultured cells [35] and stimulates topoisomerase II-mediated DNA cleavage *in vitro* [12]. The ability of NetGA to stimulate topoisomerase II-mediated DNA cleavage was tested comparatively with m-AMSA by following the conversion of pSP65 DNA into the linear form which migrates faster in polyacrylamide gels [36]. Figure 3 shows that both compounds are equally potent in inducing DNA cleavage in the 0–8 μM concentration range. At high NetGA concentration, DNA cleavage levels off and drops at a concentration of 50 μM whereas the m-AMSA-induced cleavage exhibits a continuous increase. We tested the effect of glycyl-anilino-9-aminoacridine (i.e. the intercalating moiety of the hybrid) at a concentration of 50 μM and observed no stimulation of topoisomerase II-mediated DNA cleavage (not shown). Thus, the netropsin-like moiety of NetGA plays a crucial role in stabilizing the cleavable complex.

Cytostatic and cytotoxic activities in vitro

Two different cell lines were used to evaluate the potential antitumour activity of NetGA: L1210 leukemia cells and MCF7 human mammary carcinoma cells, i.e. two cell lines derived from a leukemia and a solid tumour, respectively.

L1210 cells were exposed to NetGA for a period of up to 96 hr. The data presented in Fig. 4 indicate that the exposure to 0.5 μM NetGA for 4 days was sufficient to cause marked (i.e. 50%) inhibition of growth but weakly decreased cell viability (95% of

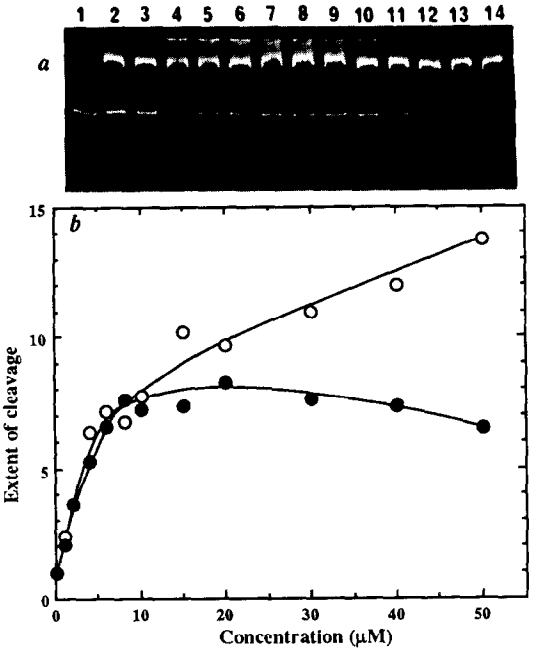

Fig. 3. Stimulation by NetGA and m-AMSA of DNA topoisomerase II-mediated double strand DNA cleavage. (a) Polyacrylamide gel electrophoresis of pSP65 DNA after cleavage in the presence of 100 μM VP-16 (lanes 2–3) or Net GA at concentrations of 50, 40, 30, 20, 15, 10, 6, 4, 2 and 1 μM (lanes 4–13), respectively, or in the absence of drug (lane 14); 25 ng of linear pSP65 DNA was run as a standard (lane 1). (b) The extent of cleavage (%) of pSP65 DNA was determined for NetGA (●) and m-AMSA (○) as described in Materials and Methods and the results expressed relative to cleavage observed in the presence of 100 μM VP-16.

the cells remained viable with 0.5 μM NetGA). Complete growth inhibition was achieved after exposure to at least 5 μM, resulting in total cell numbers of 40 and 20% of control at 48 and 96 hr, respectively, with more than 50% of the cells remaining viable in both measurements. Complete cell death was obtained only for concentrations up to 25 μM. After 48 hr exposure to 10 μM NetGA, 50% of the cells were dead and continuous exposure for up to 96 hr did not increase significantly the percentage of non-viable L1210 cells. The hybrid drug NetGA thus appears to be a potent cytostatic agent but only weakly cytotoxic.

For comparison, the reference drug m-AMSA was tested under similar conditions and 50% growth inhibition was obtained with a concentration of 0.075 μM (after 96 hr exposure, 100% inhibition obtained with 0.5 μM m-AMSA). However, if m-AMSA, as expected, is a powerful growth inhibitor for leukemic cells, it is also a toxic agent. After 96 hr exposure, a concentration of 0.5 μM m-AMSA was sufficient to kill 50% of the cells (100% with 5 μM) (not shown). Therefore, the concentration of m-AMSA which kills 50% of L1210 cells (0.5 μM) is 6–7-fold greater than the concentration which inhibits growth by 50% (75 nM). Opposingly, with NetGA it should be noted that the difference is higher since

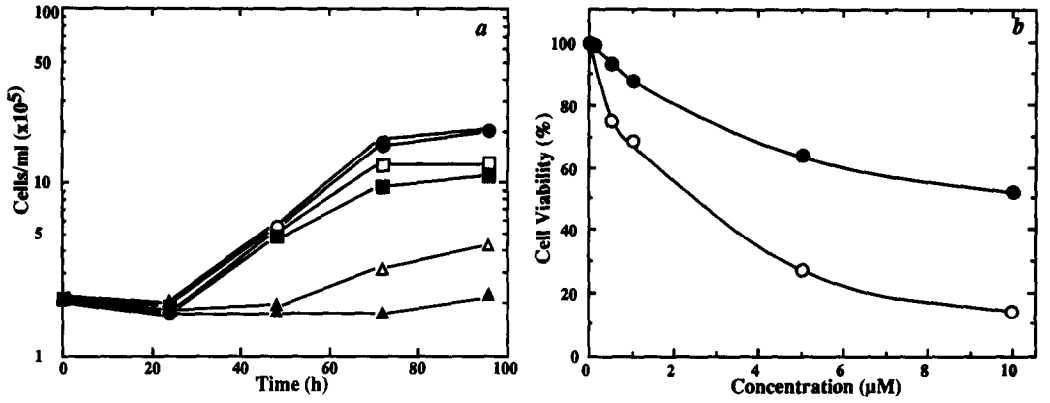

Fig. 4. Effect of different concentrations of NetGA on the growth of L1210 leukemia cells: (○) control, (●) 0.1 μM, (□) 0.5 μM, (■) 1 μM, (△) 5 μM, and (▲) 10 μM. Exponentially growing cells (0.25×10^6) were incubated with the drug for 4 days without renewal of the medium. (b) Effect of different concentrations of (●) NetGA and (○) m-AMSA on the viability of L1210 cells. Cells were incubated for 48 hr at the indicated concentration and viability was evaluated by trypan blue exclusion. Viability is expressed as a percentage relative to the control untreated cells. Values are the results of three separate determinations.

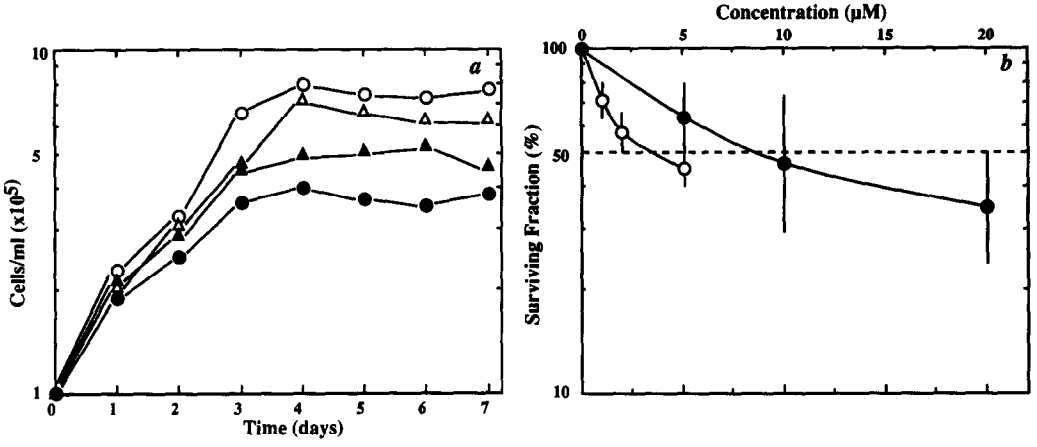

Fig. 5. (a) Growth inhibition of MCF7 cells incubated with various concentrations of NetGA: (○) control, (△) 5 μM, (▲) 10 μM, (●) 20 μM. Exponentially growing cells (1.5×10^5) were incubated with the drug for 8 days without renewal of the medium. (b) Dose–survival curves obtained in a clonogenic assay with MCF7 mammary carcinoma cells in the presence of NetGA (●) and m-AMSA (○). Vertical bars indicate SEM. Each point represents a minimum number of five observations.

the concentration giving 50% of dead cells (7.5 μM) is 15-fold greater than the concentration producing 50% growth inhibition (0.5 μM). However, it is clear that NetGA is less active than m-AMSA on L1210 cells.

With MCF7 cells, NetGA is less active by far than with leukemic cells. As shown in Fig. 5a, concentrations as high as 20 μM were required to halve the cell growth. NetGA influenced the cell proliferation in a dose-dependent manner but the percentage of growth inhibition remained relatively constant from day 1 to 7. The decrease in viability, determined by colony-forming ability, was also dose-dependent (Fig. 5b). The LD_{50} is estimated to be 10 μM for NetGA while, under identical conditions, m-AMSA shows a LD_{50} of 3.3 μM. Thus, both the cytostatic and cytotoxic activities of NetGA are lower with MCF7 cells.

Morphological cellular alterations

With both L1210 and MCF7 cells exposed continuously to a concentration of NetGA which inhibits growth by 40–60% but which is weakly toxic, we observed the presence of numerous cells of an increased size (Fig. 6). Such cellular modification is frequently observed and characterizes many antitumour drugs; for example, this phenomenon was well described with an ellipticine derivative [37]. However, more surprisingly, within these giant cells a large proportion were multinucleated (binucleated,

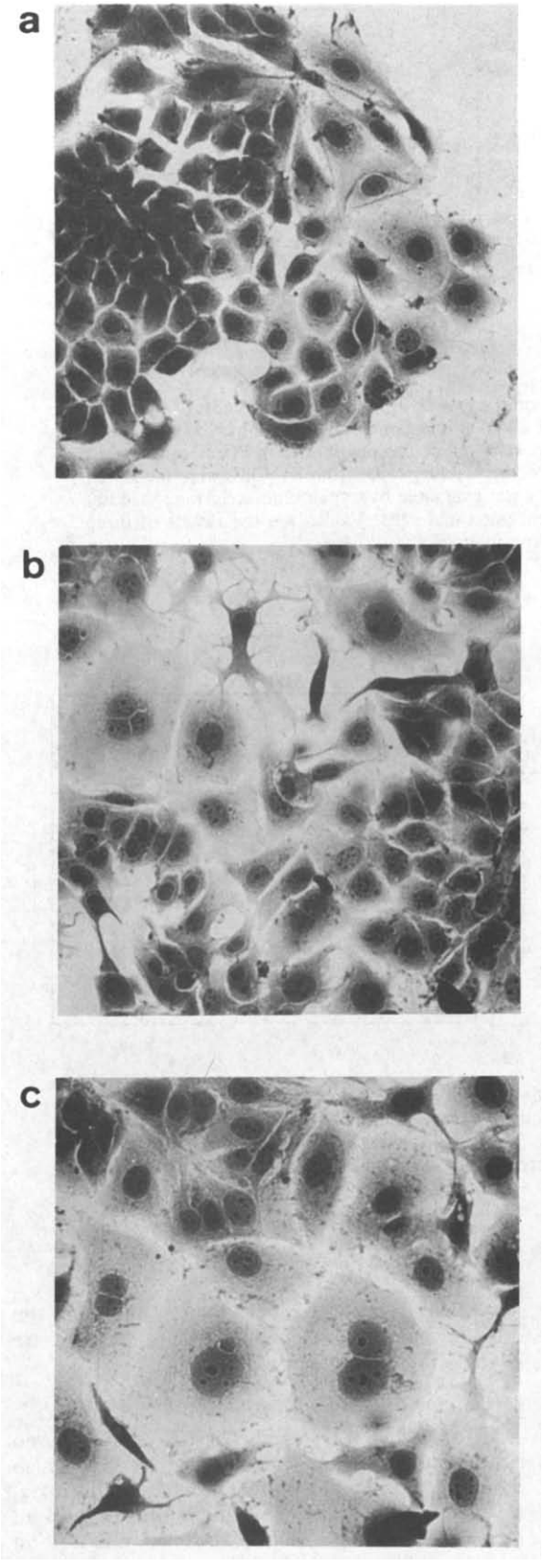

Fig. 6. Effects of NetGA on cell morphology (×100). (a) MCF7 normal cells; (b and c) hypertrophied MCF7 cells treated with 20 μM and 40 μM NetGA. Polynucleated cells are indicated by arrows.

rarely trinucleated). Binucleated cells can originate either from the failure of cytoplasmic division immediately following nuclear division (the most likely explanation) or from the fusion of two or more adjacent cells into one single cytoplasmic unit. In order to determine the process leading to these cellular alterations, we followed the cells treated with NetGA by time-lapse cinematography. MCF7 cells, which grow in a monolayer and for which the hyperplasia was particularly obvious, were used.

We observed unambiguously that hypertrophic multinuclear cells resulted from fusion between neighbouring cells. These cells were not capable of reproduction and remained viable in the culture for a limited time only. As far as we are aware, such a cellular phenomenon has never been reported with DNA-binding ligands. The significance of these cell fusions is presently unknown. However, as the structure and composition of the cell membrane play an important role in cell fusion, one could ask about the effect of NetGA on cell membrane.

Toxicity studies

Toxicity studies of NetGA were performed by the i.p. route. By this route, this agent has not revealed any type of cytotoxicity to healthy mice even at a dose as high as 50 mg/kg/day. Animal weights, aspects and behaviour were identical to those of the controls. Histology studies of the animals killed at 30 days have not shown any organ damage. Therefore, NetGA, that fared well in the *in vitro* and toxicity studies was screened for *in vivo* activity against P388 leukemia.

Antitumour activity in vivo

To test the antitumour potency of NetGA, P388 leukemia cells were inoculated i.p. in DBA2 mice and grown. According to the National Cancer Institute, leukemia P388 is a reasonable prescreen on the basis of its sensitivity to most classes of clinically effective drugs. The control mice began to die at day 10 while the leukemia-free DBA2 mice treated with NetGA under similar conditions behaved like the untreated non-leukemic DBA2 mice, thus confirming that the drug is not toxic. Leukemia sensitivity to NetGA is shown in Fig. 7 together with the results obtained with 5-fluorouracil, one of the most efficient and frequently used antitumour drugs. At 25 mg/kg/day of NetGA (repeated daily injection of days 1–5), a noticeable increase in life span was observed, expressed by a T/C of 132%. At a higher dosage (50 mg/kg/day, a non toxic dose) the effect was not improved. The observed activity corresponds to the lower limit of the significant effect on P388 in mice. With 5-fluorouracil, used as a positive control, a T/C of 154 was measured and validates the assay. m-AMSA was not tested here but the literature reports an increase in lifespan of 60 and 78% (i.e. T/C of 160 and 178) at 5.9 mg/kg/dose [38] and 8.9 mg/kg/dose [39], respectively, under comparable (but not strictly identical) conditions as for P388 cultures.

DISCUSSION

The netropsin–anilinoacridine hybrid molecule

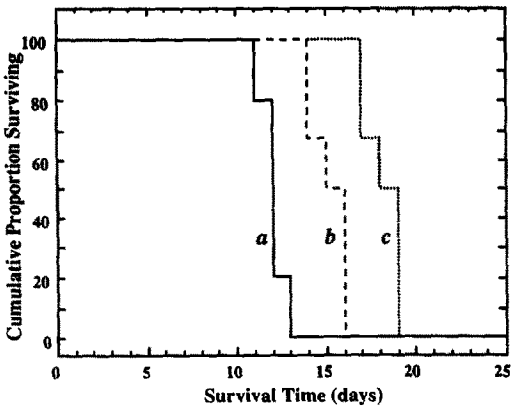

Fig. 7. *In vivo* antitumour effect of NetGA (b) at 25 mg/kg/day (N = 6) compared to 5-fluorouracil (c) at 20 mg/kg/day (N = 6), relative to untreated animals (a) (N = 5). The drug is administered on days 1–5 after tumour inoculation on DBA2 mice.

NetGA was shown recently to interact strongly with DNA in a sequence-selective manner [26]. On the basis of complementary physicochemical data, it was established that the DNA binding mechanism of this drug involves two distinct simultaneous processes: intercalation of the acridine chromophore between DNA base pairs and minor groove binding of the netropsin skeleton. Footprinting experiments revealed that the drug has retained the AT selectivity of the netropsin half of the molecule.

This mode of binding was determined with purified DNA but the major part of DNA in eukaryotic cells is packed into a nucleosomal chromatin fibre structure [40]. This higher order of structure can affect drug accessibility to DNA. Therefore, the study of the interaction of NetGA with chromatin may be more representative of the binding process that takes place *in vivo*. This interaction was studied by an electro-optical technique particularly adapted for such bifunctional ligands. Indeed, by selectively measuring the reduced linear dichroism of the acridine chromophore and the netropsin moiety in their respective absorption regions as compared to that of the bases, we can fully appreciate the orientation of the drug bound to DNA.

As the hybrid bears an anilino-aminoacridine chromophore, it is supposed to intercalate into the DNA helix. However, we showed previously that, in the presence of DNA, only a partial intercalation of the acridine plane of the hybrid is possible due to the tight anchorage of the netropsin moiety into the minor groove. When chromatin is interacting with NetGA, the negative dichroism in the 430 nm band (−0.12) is similar to that observed for the naked DNA–NetGA complex in this band, but more negative than that of chromatin at 260 nm (−0.08). This supports the view that the presence of histones in chromatin does not disturb the acridine ring intercalation, mostly influenced by the netropsin moiety of the hybrid. As regards this last moiety, it appears that the presence of histones does not modify appreciably its binding to DNA. Indeed, chromatin–NetGA and DNA–NetGA complexes have similar positive dichroism values at 310 nm. In other words, the minor groove is equally accessible in chromatin and naked DNA for this ligand. This is not surprising since circular dichroism measurements have evidenced that 80–90% of the DNA is still accessible to the minor groove binder distamycin in chromatin fibres [41]. The binding of netropsin and distamycin to chromatin takes place preferentially, if not exclusively, in the DNA linker regions and results in an increase in the internucleosomal distance [42]. Intercalators also bind to the internucleosomal region of DNA at high DNA-phosphate over ligands ratios [43]. On the basis of these references and as the ELD signals always displayed perfectly normal shapes, we can assume that NetGA binds to linker DNA too.

Inhibition of replication and transcription processes by drug binding to DNA and chromatin could be directly responsible for the cytotoxic effect observed. However, although it is known that DNA binding is a necessary condition for cytotoxicity, the different activities of anilino-acridines derivatives which all intercalate and exhibit similar affinities for DNA have not been explained [17].

Many studies have indicated that topoisomerase II is a privileged cellular target through which a large number of efficient anticancer drugs produce their cytotoxic effects. Correlation between topoisomerase II-mediated DNA cleavage and cytotocixity has been observed for drugs such as anthracyclines [44] and acridines including m-AMSA [18, 45, 46]. Moreover, supporting this finding further are the observations that: (1) alteration of the topoisomerase II enzyme structure affects the cytotoxicity of m-AMSA and analogues and (2) cells resistant to m-AMSA present altered topoisomerase II function [38, 47, 48]. Thus, the effect of NetGA on the functional activity of this enzyme needed to be investigated.

The alteration of the functional activity of topoisomerase II, checked *in vitro* by the occurrence of linear DNA representative of the stabilization of the cleavable complex, is observed with NetGA. The absence of effect of the glycyl-anilino-9-aminoacridine chromophore (i.e. the intercalating moiety of the hybrid) compared to the marked effect of the hybrid suggests that the netropsin skeleton of the hybrid undergoes strong interaction with the enzyme–DNA complex. With naked DNA as well as with DNA associated in a nucleosomal structure, the netropsin moiety was found anchored perfectly to the minor groove of the DNA. Therefore, both moieties of the hybrid appear as determinants for the interaction with the topoisomerase II–DNA complex.

In order to propose a scheme by which the drug can alter the topoisomerase II activity, it is worth remembering the present state of knowledge of the postulated mechanism of the interaction of m-AMSA with the topoisomerase II–DNA complex.

Intercalation of m-AMSA (with a DNA unwinding angle of 20.5° [49]) occurs through the acridine ring and projects the anilino ring from one of the grooves of the DNA; the minor groove was proposed initially [50] but major groove binding has been postulated

recently [51]. Modification of the acridine ring results in altered DNA binding properties [52]. Opposingly, studies with m-AMSA analogues for which the acridine part is held constant while the anilino substituents are changed, have shown that the cytotoxicity varies almost independently of the DNA association constant [47, 53]. Furthermore, depending on the structure of the m-AMSA derivative, the efficiency of the formation of the cleavable complex can vary widely with little dependence on DNA binding [17, 46]. With respect to these considerations, it is very likely that action on topoisomerase II is directly responsible for the biological activity of the drug and that m-AMSA has both a DNA-binding domain (the acridine nucleus) and a protein-binding domain (the anilino side chain) [39]. However, a direct interaction between the enzyme and m-AMSA has not been reported. Similar topoisomerase II–DNA binding models, that divides the drug into two functional domains, were also proposed to explain the stabilizing effect of anthracyclines and epipodophyllotoxins [54].

If the analogy between m-AMSA and NetGA is correct, one could predict that the enzyme-binding domain of the hybrid is the pseudopeptide netropsin moiety. This hypothesis may be retained because of the comparable interaction of both drugs with topoisomerase II–DNA complexes. However, it cannot be stated that the stabilizing effect is due specifically to the bispyrrole entity; it may be due to the bulky, positively charged netropsin moiety attached to the aniline. Indeed, in a series of acridine derivatives, the one bearing the bulkiest substituent (SN 12489, an analogue bearing two consecutively linked aniline rings) was found to be the most potent topoisomerase II inhibitor [17, 46]. However, the fact that groove binders also affect the topoisomerase II activity certainly needs to be considered here as well as the size of the bispyrrole moiety of NetGA.

It was shown recently that distamycin stimulates substantially the topoisomerase II-catalysed relaxation of supercoiled DNA plasmid [21, 23] and inhibits the formation of double strand breaks in intact nuclei [22]. Although the exact mechanism of interaction between distamycin and the amsacrine-stabilized topoisomerase II–DNA cleavable complex is totally unknown, these different effects were correlated with the AT sequence specificity of distamycin and attributed to a redistribution of the enzyme along the DNA sequence and alteration of the DNA structure upon distamycin binding.

Considering both these observations and our results (Fig. 3), it is tempting to speculate that the netropsin-like half of the hybrid acts as distamycin does by preventing the trapping of the cleavable complex by the m-AMSA-like half of NetGA. This effect is apparent at drug concentrations above 10 μM leading to a concentration–response curve reminiscent in shape of those obtained with other intercalating agents [55].

Before discussing the biological activity of NetGA, it is worth remembering here that the covalent linkage of an acridine chromophore to a netropsin one is particularly well justified not only on grounds of DNA binding due to their base-specific complementarity (GC [56] and ATAT [57], respectively) but also because of the crucial role played by acridine in cellular transport. Both netropsin and 9-amino-acridine exhibit a nuclear binding preference but with slow and fast kinetics of cell penetration [58, 59], respectively. With the hybrid NetGA, the acridine chromophore speeds up the rate of penetration of netropsin and acts as a vector allowing a rapid accumulation of the drug into the nucleus [60].

The combination of groove binders with m-AMSA derivatives (and intercalating agents in general) not only raises questions about mechanism, but also suggests possibilities for therapeutic application. The present results show that NetGA is less active than m-AMSA both *in vitro* and *in vivo* on the above selected models but reveals an interesting biological capacity. Indeed, the linking of the netropsin fragment and the anilino-aminoacridine, both individually inactive *in vivo*, affords a drug with marked cytostatic properties against L1210 cells and a moderate but noticeable antitumour effect *in vivo*. The lower activity against MCF7 cells is not surprising and can be connected with the inactivity of m-AMSA against solid tumours such as melanomes, breast and lung cancers. m-AMSA is very useful in the treatment of refractory acute myelogenous leukemia [61, 62].

To sum up, this netropsin–acridine hybrid has detectable biochemical and antitumour activities while the individual components do not. The biological properties of NetGA can be considered as the results of a tight interaction with DNA and inhibition of topoisomerase II; of course, other associated targets may be involved. Linkage of anilino-aminoacridine to netropsin appears to have the same qualitative effects on the anti-topoisomerase II activity of this molecule as does the substitution (i.e. *m*-methoxy) which yield m-AMSA. Elucidation of the mechanisms of these effects will be a key area for future research of tumour active drug.

More active amsacrine analogues (substituted at position 4 of the acridine ring by a carboxamide group) have been designed and have justified clinical trials [63]. Highly active antileukemic drugs already exist but show often intense intrinsic toxicity. As an illustrative example, it can be mentioned that m-AMSA itself is very toxic; the treatment of non-leukemic mice with 20 mg/kg/dose of m-AMSA on an intermittent schedule killed 100% of the treated mice [38]. Evaluation of the therapeutic potential of a drug necessitates the consideration of both the activity and toxicity of the tested drug. NetGA exhibits a moderate activity *in vivo* but is considerably less toxic (if toxic at all) than m-AMSA *in vivo*. Therefore, we think that it could be interesting to develop other related ligands in an attempt to obtain more active and weakly cytotoxic hybrids. The concept of hybrid ligand deserves thus to be retained.

The success of this first hybrid has encouraged us to design other related compounds. Firstly, we have synthesized conjugates between a distamycin fragment and ellipticine-intercalating chromophores, in order to find drugs with better therapeutic efficiency against solid tumours. It should be noted here that one of the approaches followed to find m-AMSA analogues with different spectra of activity consists in the combination of m-AMSA derivatives

with DNA-alkylating groups, i.e. to develop hybrid ligands [64, 65]. Secondly, we elaborated recently hybrids between netropsin and tumour active m-AMSA-4-carboxamide derivatives. These derivatives bear the methoxy and methanesulphonamide substituents on the aniline ring which restore the oxidizable properties. Oxidative activities should contribute to an additional mechanism of toxicity. In other words, the replacement of the anilino-acridine part of NetGA by a tumour active amsacrine analogue may lead to more efficient hybrid molecules. Therefore interesting development can be expected to take place in this burgeoning new area of anticancer drug pharmacology.

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REFERENCES

1. Waring MJ, DNA modification and cancer. *Annu Rev Biochem* **50**: 159–192, 1981.
2. Hurley LH, DNA and associated targets for drug design. *J Med Chem* **32**: 2027–2033, 1989.
3. Dabrowiak JC and Goodisman J, Quantitative footprinting analysis of drug–DNA interactions. In: *Chemistry and Physics of DNA–Ligand Interactions* (Ed. Kallenbach NR), pp. 143–174. Adenine Press, New York, 1989.
4. Nielsen PE, Sequence-selective DNA recognition by synthetic ligands. *Bioconjugate Chem* **2**: 1–12, 1991.
5. Lown JW, Molecular mechanisms of DNA sequence recognition by groove binding ligands: biochemical and biological consequences. In: *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions* (Eds. Pullman B and Jortner J), pp. 103–122. Kluwer Academic, Netherlands, 1990.
6. Lown JW, Lexitropsins: rational design of DNA sequence reading agents as novel anti-cancer agents and potential cellular probes. *Anti-Cancer Drug Dis* **3**: 25–40, 1988.
7. Denny WA, DNA-intercalating ligands as anti-cancer drugs: prospects for future design. *Anti-Cancer Drug Des* **4**: 241–263, 1989.
8. Baguley BC, DNA intercalating anti-tumour agents. *Anti-Cancer Drug Dis* **6**: 1–35, 1991.
9. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **54**: 665–697, 1985.
10. Maxwell A and Gellert M, Mechanistic aspects of DNA topoisomerases. *Adv Protein Chem* **38**: 69–107, 1986.
11. Sutcliffe JA, Gootz TD and Barrett JF, Biochemical characteristics and physiological significance of major DNA topoisomerases. *Antimicrob Agents Chemother* **33**: 2027–2033, 1989.
12. Nelson EM, Tewey KM and Liu LF, Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. *Proc Natl Acad Sci USA* **81**: 1361–1365, 1984.
13. Lock RB and Ross WE, DNA topoisomerases in cancer therapy. *Anti-Cancer Drug Design* **2**: 151–164, 1987.
14. Kohn KW, Pommier Y, Kerrigan D, Markovits J and Covey JM, Topoisomerase II as a target of anticancer drug action in mammalian cells. *Natl Cancer Inst Monogr* **4**: 61–71, 1987.
15. Drlica K and Franco RJ, Inhibitors of DNA topoisomerases. *Biochemistry* **27**: 2253–2259, 1988.
16. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
17. Pommier Y, Covey J, Kerrigan D, Mattes W, Markovits J and Kohn KW, Role of DNA intercalation in the inhibition of purified mouse leukemia (L1210) DNA topoisomerase II by 9-aminoacridines. *Biochem Pharmacol* **36**: 3477–3486, 1987.
18. Schneider E, Darkin SJ, Lawson PA, Ching LM, Ralph RK and Baguley BC, Cell line selectivity and DNA breakage properties of the antitumour agent *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; role of DNA topoisomerase II. *Eur J Cancer Clin Oncol* **24**: 1783–1790, 1988.
19. McHugh MM, Woynarowski JM, Sigmund RD and Beerman TA, Effects of minor groove binding drugs on mammalian topoisomerase I activity. *Biochem Pharmacol* **38**: 2323–2328, 1989.
20. McHugh MM, Sigmund RD and Beerman TA, Effects of minor groove binding drugs on camptothecin-induced DNA lesions in L1210 nuclei. *Biochem Pharmacol* **39**: 707–714, 1990.
21. Woynarowski JM, McHugh MM, Sigmund RD and Beerman TA, Modulation of topoisomerase II catalytic activity by DNA minor groove binding agents distamycin, Hoechst 33258, and 4′,6-diamidine-2-phenylindole. *Mol Pharmacol* **35**: 177–182, 1989.
22. Woynarowski JM, Sigmund RD and Beerman TA, DNA Minor groove binding agents interfere with topoisomerase II mediated lesions induced by epipodophyllotoxin derivative VM-26 and acridine derivative m-AMSA in nuclei from L1210 cells. *Biochemistry* **28**: 3850–3855, 1989.
23. Fesen M and Pommier Y, Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamycin in Simian virus 40 DNA. *J Biol Chem* **264**: 11354–11359, 1989.
24. Bailly C and Hénichart JP, DNA-recognition by intercalator-minor groove binder hybrid molecules. *Bioconjugate Chem*, in press.
25. Bailly C, Pommery N, Houssin R and Hénichart JP, Design, synthesis, DNA-binding and biological activity of a series of DNA minor groove binding intercalating drugs. *J Pharm Sci* **78**: 910–917, 1989.
26. Bailly C, Helbecque N, Hénichart JP, Colson P, Houssier C, Rao KE, Shea RG and Lown JW, Molecular recognition between oligopeptides and nucleic acids. DNA sequence specificity and binding properties of an acridine-linked netropsin hybrid ligand. *J Mol Recognit* **3**: 26–35, 1990.
27. Hénichart JP, Bernier JL and Catteau JP, Interaction of 4-(9-acridinylamino)aniline and derivatives with DNA, Influence of a lysyl glycyl side chain on the binding parameters. *Hoppe Seylers Z Physiol Chem* **363**: 835–841, 1982.
28. Cain BF, Atwell GJ and Denny WA, Potential antitumour agents 4′-(acridin-9-ylamino)methanesulfon-anilides. *J Med Chem* **18**: 1110–1117, 1975.
29. Marquet R, Colson P, Matton AM, Houssier C, Thiry M and Goessens G, Comparative study of the condensation of chicken erythrocyte and calf thymus chromatins by di- and multivalent cations. *J Biomol Struct Dyn* **5**: 839–857, 1988.
30. Miller KG, Liu LF and Englund PT, A homogeneous type II DNA topoisomerase from HeLa cell nuclei. *J Biol Chem* **256**: 9334–9339, 1981.
31. Halligan BD, Edwards KA and Liu LF, Purification and characterization of a type II DNA topoisomerase from bovine calf thymus. *J Biol Chem* **260**: 2475–2482, 1985.
32. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982.
33. Puck TT and Marcus PI, A rapid method for viable

cell titration and clone production with Hela cells in tissue culture: the use of X irradiated cells to supply conditioning factors. *Proc Natl Acad Sci USA* **41**: 432–437, 1955.

34. Hagmar P, Marquet R, Colson P, Kubista M, Nielsen P, Norden B and Houssier C, Electric and flow linear dichroism of unfolded and condensed chromatin: a comparative study at low and intermediate ionic strength. *J Biomol Struct Dyn* **7**: 19–33, 1989.
35. Zwelling LA, Kerrigan D and Michaels S, Cytotoxicity and DNA strand breaks by 5-iminodaunorubicin in mouse leukemia L1210 cells: comparison with adriamycin and 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. *Cancer Res* **42**: 2687–2691, 1982.
36. Monnot M, Mauffret O, Simon V, Lescot E, Psaume B, Saucier J-M, Charra M, Belheradek Jr J and Fermandjian S, DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. A three-mode binding model for ellipticinium derivatives. *J Biol Chem* **266**: 1820–1829, 1991.
37. Vilarem MJ, Gras MP, Rivalle C and Larsen CJ, Possible relationship between pyridopyrroloisoquinoline derivative (BD-40) cell uptake and cell viability. *Biochimie* **64**: 923–932, 1982.
38. Finlay GJ, Wilson WR and Baguley BC, Chemoprotection by 9-aminoacridine derivatives against the cytotoxicity of topoisomerase II-directed drugs. *Eur J Cancer Clin Oncol* **25**: 1695–1701, 1989.
39. Baguley BC, Holdaway KM and Fray LM, Design of DNA intercalators to overcome topoisomerase II-mediated multidrug resistance. *J Natl Cancer Inst* **82**: 398–402, 1990.
40. Felsenfeld G and McGhee JD, Structure of the 30 mm chromatin fiber *Cell* **44**: 375–377, 1986.
41. Zimmer C and Wahnert U, Non-intercalating DNA-binding ligands: specificity of the interaction and there use as tools in biophysical, biochemical and biological investigations of the genetic material. *Prog Biophys Mol Biol* **47**: 31–112, 1986.
42. Koch MHJ, Sayers Z, Vega MC and Michon AM, The superstructure of chromatin and its condensation mechanism IV. Enzymatic digestion, thermal denaturation, effect of netropsin and distamycin. *Eur Biophys J* **15**: 133–140, 1987.
43. Sen D and Crothers DM, Influence of DNA-binding drugs on chromatin condensation. *Biochemistry* **25**: 1503–1509, 1986.
44. Zunino F and Capranico G, DNA topoisomerase II as the primary target of anti-tumor anthracyclines. *Anti-Cancer Drug Design* **5**: 307–317, 1990.
45. Covey JM, Kohn KW, Kerrigan D, Tilchen EJ and Pommier Y, Topoisomerase II-mediated DNA damage produced by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide and related acridines in L1210 cells and isolated nuclei: relation to cytotoxicity. *Cancer Res* **48**: 860–865, 1988.
46. Rowe TC, Chen GL, Hsiang YH and Liu LF, DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II. *Cancer Res* **46**: 2021–2026, 1986.
47. Pommier Y, Kerrigan D, Schwartz RE, Swack JA and McCurdy A, Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* **46**: 3075–3081, 1986.
48. Per SR, Mattern MR, Mirabelli CK, Drake FH, Johnson RK and Crooke ST, Characterization of a subline of P388 leukemia resistant to amsacrine evidence of altered topoisomerase II function. *Mol Pharmacol* **32**: 17–25, 1987.
49. Waring MJ, DNA-binding characteristics of acridinylmethanesulphoanilide drugs: comparison with antitumour properties. *Eur J Cancer* **12**: 995–1001, 1976.
50. Denny WA, Atwell GJ and Baguley BC, Potential antitumor agents. 39. Anilino ring geometry of amsacrine and derivatives: relationship to DNA binding antitumor activity. *J Med Chem* **26**: 1625–1630, 1983.
51. Wakelin LPG, Chetcuti P and Denny WA, Kinetic and equilibrium binding studies of amsacrine-4-carboxamides: a class of asymmetrical DNA-intercalating agents which bind by threading through the DNA helix. *J Med Chem* **33**: 2039–2044, 1990.
52. Baguley BC, Denny WA, Atwell GJ and Cain BF, Potential antitumour agents. 35. Quantitative relationships between antitumor (L1210) potency and DNA binding for 4′-(9-acridinylaminomethanesulphon-*m*-anisidide analogues. *J Med Chem* **24**: 520–525, 1981.
53. Baguley BC and Nash R, Antitumour activity of substituted 9-anilinoacridines: comparison of *in vivo* and *in vitro* testing systems. *Eur J Cancer* **17**: 671–679, 1981.
54. Chow KC, McDonald TL and Ross WE, DNA binding by epipodophyllotoxins and *N*-acyl anthracyclines: implications for mechanism of topoisomerase II inhibition. *Mol Pharmacol* **34**: 467–473, 1988.
55. Pommier Y, Schwartz RE, Zwelling LA and Kohn KW, Effects of DNA intercalating agents on topoisomerase II induced DNA strand cleavage in isolated mammalian cell nuclei. *Biochemistry* **24**: 6406–6410, 1985.
56. Feigon J, Denny WA, Leupin W and Kearns DR, Interactions of antitumor drugs with natural DNA: ^{1}H NMR study of binding mode and kinetics. *J Med Chem* **27**: 450–465, 1984.
57. Portugal J and Waring MJ, Comparison of binding sites in DNA for berenil, netropsin and distamycin. A footprinting study. *Eur J Biochem* **167**: 281–289, 1987.
58. Bailly C, Catteau JP, Hénichart JP, Reszka K, Shea RG, Krowicki K and Lown JW, Subcellular distribution of a niroxide spin-labeled netropsin in living KB cells. Electron paramagnetic resonance and sequence specificity studies. *Biochem Pharmacol* **38**: 1625–1630, 1989.
59. Lemay P, Bernier JL, Hénichart JP and Catteau JP, Subcellular distribution of nitroxide spin-labelled 9-aminoacridine in living KB cells. *Biochem Biophys Res Commun* **111**: 1074–1081, 1983.
60. Bailly C and Hénichart JP, Subcellular distribution of a nitroxide spin-labelled netropsin–acridine hybrid in living KB cells. Electron Spin Resonance study. *Biochem Biophys Res Commun* **167**: 798–806, 1990.
61. Zittoun R, *m*-AMSA: a review of clinical data. *Eur J Cancer Clin Oncol* **21**: 649–653, 1985.
62. Cassileth PA and Gale RP, Amsacrine: a review. *Leuk Res* **10**: 1257–1265, 1986.
63. Hardy JR, Harvey VJ, Paxton JW, Evans P, Smith S, Grove W, Grillo-lopez AJ and Baguley BC, a phase I trial of the amsacrine analog 9-[[2-methoxy-4-[(methylsulfonyl)amino]phenyl]amino]-*N*,5-dimethyl-4-acridinecarboxamide (CI-921). *Cancer Res* **48**: 6593–6596, 1988.
64. Palmer BD, Lee HH, Johnson P, Baguley BC, Wicklam G, Wakelin LPG, McFadyen WD and Denny WA, DNA-directed alkylating agents. 2. Synthesis and biological activity of platinum complexes linked to 9-anilinoacridine. *J Med Chem* **33**: 3008–3014, 1990.
65. Valu KK, Gourdie TA, Boritzki TJ, Gravatt GL, Baguley BC, Wilson WR, Wakelin LPG, Woodgate PD and Denny WA, DNA-directed alkylating agents. 3. Structure–activity relationships for acridine-linked aniline mustards: consequences of varying the length of the linker chain. *J Med Chem* **33**: 3014–3019, 1990.