Cytotoxic and Antitumor Activity of 1-Nitroacridines as an Aftereffect of Their Interstrand DNA Cross-Linking

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ABSTRACT

To determine whether the toxic effects and changes in many cell functions caused by antitumor 1-nitroacridines are related to their enzymatically mediated covalent interstrand DNA cross-linking (J. Konopa, J. W. Pawlak, and K. Pawlak. Chem.-Biol. Interact., 43: 175–197, 1983), the cross-linking potency of the derivatives with structural modifications in position 9 of the acridine nucleus was estimated as their in vitro threshold concentrations (0.3 to 4.5 μM), beyond which the first interstrand DNA cross-links could be detected in DNA of cultured HeLa S3 cells with a polyethylene glycol 6000-Dextran T500 assay. Statistically significant (p < 0.05) correlations exist between the cross-linking potency of 1-nitroacridines and their in vitro antitumor activity and toxicity against mice with Sarcoma 180 tumors in solid form (3 to 1065 μmol/kg of body weight), as well as their in vitro cytotoxicity against cultured HeLa or HeLa S3 cells (0.0005 to 7.2 μM), indicating that the interstrand DNA cross-linking potency might be one of primary determinants of in vivo and in vitro biological activity of 1-nitroacridine antineoplastic drugs. Susceptibility of the parent agents to reduction does not appear to be a rate-limiting factor of DNA cross-linking potency of 1-nitroacridines and their metabolic transformations (J. W. Pawlak, and J. Konopa. Biochem. Pharmacol., 28: 3391–3402, 1979), because no significant differences were observed among the agents with respect to their polarographic half-wave potentials estimated under anaerobic conditions.

INTRODUCTION

The search for antitumor agents among acridine derivatives done in Poland has identified as candidates the drugs of 1-nitroacridine series (6, 8, 13). Elucidation of the nature of the active 1-nitroacridine species and the lesions it produces, and the correlation of molecular processes and chemical structure with antitumor and potent cytotoxic (8, 11, 13, 19) effects of 1-nitroacridines became of obvious importance, as some of the derivatives had been introduced into clinics (6, 23). The 1-nitroacridine action in living organisms or cultured cells is apparently complex; the agents inhibit macromolecular biosyntheses (6, 19), interfere with RNA and protein metabolism (6), and induce chromosome aberrations in mammalian cells (4, 6); the 1-[14C]-nitroacridine ratio found in different cells varies (4, 6, 10, 18).

The potent cytotoxic effects of the 1-nitroacridines cannot be attributed (19) to physicochemical binding (intercalation) with a presumable target DNA (3, 9, 19), although such interaction undoubtedly occurred (3, 9, 19). We found that the 1-nitroacridines, unlike their nonantitumor isomers with the nitro group in positions 2, 3, or 4 of the acridine nucleus, required transformation(s) in the cell to express their potency (9, 10, 18). A novel type of complex metabolic activation, involving both oxidative and reductive pathways, resulted in vitro in the formation of reactive site on the acridine molecule capable of irreversible (covalent) binding of 1-nitroacridines with target macromolecules of tissue homogenates (17). This view coincided with our observation that 1-nitro-9-(3'-dimethylamino-n-propylamino)acridine, an antineoplastic drug named Ledakrin or Nitracrine5 (6, 23), actively bound covalently with DNA and other subcellular macromolecules of animals experimental tumor cells in vivo (9, 10, 18), and of cultured human tumor or bacterial cells (18). Further study showed that Ledakrin and other equally potent 1-nitroacridines cross-linked complementary strands of DNA of the latter biological systems (9, 10, 12). Ledakrin failed to bind covalently or to induce discernable interstrand cross-links when incubated with single-stranded DNAs in a cell-free system (9, 10, 12). The phenomena of Ledakrin covalent binding and cross-linking of cellular DNA have been confirmed by others with L1210 cells in vivo (4). On the contrary, the nonantitumor isomeric nitroacridines were incapable of interstrand cross-linking of the DNA of HeLa (12) or bacterial (12) cells in culture, or of Ehrlich ascites6 or L1210 tumor cells in vivo (4). Moreover, the 2- or 3-nitroacridines diminished cross-linking effects caused by Ledakrin or mitomycin C when added along with the latter agents to the HeLa cell cultures (12). Hence, the hitherto presented results could be summarized to suggest that the toxic effects and changes in many cell functions caused by antitumor 1-nitroacridines are related to the chain of sublethal and lethal events initiated by mono- or bifunctional alkylation of cellular DNA (12).

The latter hypothesis was verified in the present study by determining whether covalent (irreversible) interstrand DNA cross-linking was peculiar to highly cytotoxic 1-nitroacridines, or if it could be extended to other 1-nitroacridine derivatives exhibiting a wide disparity in their biological potency, and with various modifications in their aliphatic side-chain moiety (Table 1). Next, the correlations between DNA cross-linking potency and antitu-
mor activity and (cyto)toxicity of the agents were ascertained, and the existence of a positive and significant correlation was assumed to be a reflection of a causal relationship between the considered events. Finally, we wondered whether a susceptibility also to initiate a process of evaluating potential of nitroacridines in vitro under anaerobic conditions, and related these values to their DNA cross-linking potency and their metabolic transforma-
tions (17), thus helping us to understand biochemical mecha-
nisms of DNA cross-linking by 1-nitroacridines, and of formation of particular acridine-DNA component adducts in vivo, as the structures of the latter have been indicated (12). Hence, we determined polarographic half-wave potentials of 1-nitroacridine in vitro under anaerobic conditions, and related these values to their in vivo and in vitro biological activity and their in vitro DNA cross-linking potency. The polarographic analyses were done also to initiate a process of evaluating potential of nitroacridines as adjuvants to cancer radiotherapy.

MATERIALS AND METHODS

Nitroacridines. The 1-nitroacridine derivatives (Table 1) were synthe-
sized as mono- or dihydrochlorides in our Department and their purity was ascertained by thin-layer chromatography (19). The agents were dissolved just before their addition to cell cultures in distilled water, except for Compounds 2, 4, 14, and 16 to 19 (cf. Table 1 for 1-nitroacridine code numbers) which were dissolved in dimethyl sulfoxide and diluted in distilled water; fresh nitroacridine solutions were prepared for each experiment. Agents were added in volumes equal to 0.25% that of the cell cultures.

Cells. HeLa (12, 18, 19) or HeLa S3 (Grand Island Biological Co., Grand Island, NY) cells in log-phase growth in monolayer culture on glass were maintained in controlled air-5% CO2 humidified atmosphere at 37° in Eagle’s minimal essential medium (in Hanks’ balanced salt solution) or in Joklik’s modified essential medium (50 mM HEPES, pH 7.4). The cytototoxic activities of the agents tested were defined as their in vitro concentrations causing 50% inhibition of 72-hr growth, and they were determined from dose-response curves in semilogarithmic scale (19).

In Vitro Cytotoxicity Assays. Cytotoxic activities of 1-nitroacridine derivatives against HeLa cell lines were determined by the method of Smith et al. (20) with modifications, the details of which have been described elsewhere (12). The cytototoxic activities (the ED50 values) of the agents tested were defined as their in vitro concentrations causing 50% inhibition of 72-hr growth, and they were determined from dose-response curves in semilogarithmic scale (19).

Treatment of HeLa S3 Cells with 1-Nitroacridines and Estimation of Chemically Induced Interstrand DNA Cross-Linking. HeLa S3 cells (1.5 X 10⁶) were plated in 20-cm glass Leighton tubes (Belco Glass, Inc., Vineland, NJ) containing 10 ml of Joklik’s modified essential medium and were cultivated for 24 hr. The medium was replaced with a fresh one containing [methyl-3H]thymidine 1 µCi/ml; specific activity, 18.6 Ci/mmol; UVVR, Prague, Czechoslovakia) and the growth was continued for a further 24 hr. Then, the cells (5 X 10⁶) were exposed to the 1-nitroacridines for 24 hr at 37°, and the cross-linking reaction was terminated by removing the medium and washing the cells twice with 10 ml of ice-cold 0.9% (w/v) NaCl solution. Total intracellular HeLa S3 DNA was isolated by the proteinase K (Merck Co., Darmstadt, West Germany)-SDS method of Gross-Belard et al. (7) with modifications, the details of which are given hereafter. The collected cells (12) were lysed with 4 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 10 mM NaCl, 0.5% SDS, and proteinase K (50 µg/ml). DNA was deproteinized twice with an equal volume of phenol (distilled and saturated with 500 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 10 mM NaCl, and 0.5% SDS), followed by extraction of the aqueous phase with chloroform. Dialyses were carried out in pretreated (14) Visking dialysis tubing 20/32 (Union Carbide Corp., Chicago, IL), using the thin-layer dialysis technique (12). RNase solutions were heated prior to use at 80° for 10 min to destroy any possible contaminating DNase activity. The final DNA samples, obtained in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 10 mM NaCl, were dialyzed against 10 mM potassium phosphate-2 mM Na₂HPO₄ buffer (pH 7.0) until the complete exchange of the retained material (12).

Chemically induced and/or naturally occurring cross-linked DNA mol-
eules were detected by their ability to survive denaturing treatments by returning at once to their original bihelical form when the denaturing conditions were removed, as such a procedure applied to noncross-linked DNA is an irreversible process, resulting in disordered single-stranded molecules. The 2-phase system using dextran and PEG was applied to partition DNA species according to their bihelical or denatured configuration (12). After optimization, calibrations of phase system assays done according to the protocol previously described (12), the routine phase system assay for chemically cross-linked HeLa S3 DNA was established; DNA was denatured by heat by placing sealed vials containing dialyzed DNA solutions [50 to 100 µg DNA/ml of 10 mM potassium phosphate-2 mM Na₂HPO₄ buffer (pH 7.0)] into boiling water for 10 min, followed by quenching in ice water. Then, 3-ml DNA samples were sonicated for 90 sec at 0° and 120 watt with a Branson B-12 Sonifier (Branson Sonic Power Co., Danbury, CT) and warmed to 20-25°, after which 1.0-ml aliquots of DNA solutions were transferred to 15-mm diameter x 35-mm screw-cap, glass round-bottomed tubes. To each DNA sample, 0.55 ml of carefully emulsified polymer stock containing 16.8% (w/v) dextran (Dextran T500, Lot 4094; Pharmacia Fine Chemicals AB, Uppsala, Sweden), and 9.2% (w/v) PEG (PEG Lot 16C-00603; Sigma Chemical Co., St. Louis, MO) in 10 mM potassium phosphate-2 mM Na₂HPO₄ buffer (pH 7.0) was added by means of a 1-ml tuberculin syringe. The system was mixed vigorously at 20° for 20 min, and the mixture was centrifuged at 800 x g for 10 min at 20° in a swinging-bucket rotor to separate the phases. The 100-µl aliquot of the upper PEG-rich phase was removed, placed in a glass scintillation vial, and DNA was hydrolyzed with 1 ml of 5.5% (w/v) perchloric acid by exposure to 80° for 40 min. Samples were then counted for radioactivity in 10 ml of Tritosol (5) scintillation fluid in Isocap Model 300 liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, IL) with accuracy of ±1%. Radioactivity was corrected for quenching by sample channels ratio standardization; the counting efficiency amounted to 9 to 14%. The 100-µl aliquots of the DNA solution prior to its partitioning in the phase system were hydrolyzed and counted for radioactivity in the manner just de-
scribed.

Percentage of chemically imposed cross-linked DNA molecules was calculated from the difference between recovery in the upper phase of the DNA isolated from 1-nitroacridine-treated and untreated cells, as reported elsewhere (12). Recoveries of particular DNA species were estimated from the total radioactivities of DNA samples before and after partitioning in phase system, necessary corrections for the denatured DNA that entered the upper phase were made (12). Routinely, it was possible to partition by means of a single extraction step about 78.5% of bihelical (native) DNA into the upper (PEG-rich) phase and about 97.5% of the single-stranded (denatured) DNA into the opposite (dextran-rich) phase.

The method for determination of chemically cross-linked DNA mole-
cules (12), as it is now applied, clearly distinguishes between interstrand DNA cross-links and DNA-protein cross-links, since the latter type of cross-links is greatly reduced by treatment of the cell lysate with proteinase K in the course of intracellular DNA isolation.

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### Table 1

<table>
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<th>R₂</th>
<th>Other</th>
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<td>C-849 (NH(CH₃)₁₅CH₃OH)</td>
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<td>C-867 (NH(CH₃)₁₅CH₃)</td>
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* Antineoplastic drug named Ledakrin (cf. Ref. 6) or Nitracrine (WHO name; cf. Ref. 23).

In Vivo Antitumor Activity Estimates. The in vivo antitumor activities (the **AAM** values) of 1-nitroacridine derivatives were determined as their doses causing 50% inhibition of Sarcoma 180 tumor growth in mice (8). Starting the next day after transplantation of Sarcoma 180 solid tumor, mice received i.p. 5 to 6 daily doses of the agent. On the eighth day the percentage of tumor growth inhibition was determined from a difference between the mean weight of the tumor in control mice (18 animals) and that of treated mice (7 animals). The **AAM** values were determined by a least-squares method from dose-response dependence in semilogarithmic scale. The error of estimate of log **AAM** is ±0.15 that which imposes the **AAM** confidence interval of (**AAM**/1.4) ± (**AAM** x 1.4); for experimental details see Ref. 8; the **AAM** values are those in Ref. 8, except for the values indicated in Table 2 of this paper.

In Vivo Toxicity Estimates. The in vivo toxicities (the **TD₅₀** values) of 1-nitroacridine derivatives were estimated as their doses causing death of one-half of the population of mice treated with the agents. Mortality of the animals was monitored throughout 8 days of the regimen, and the mice dead before the eighth day were excluded from antitumor activity determinations (8). The error of estimate of log **TD₅₀** is ±0.17 that which imposes the **TD₅₀** confidence interval of (**TD₅₀**/1.5) ± (**TD₅₀** x 1.5); for the experimental details see Ref. 8; the **TD₅₀** values are those in Ref. 8 except for those indicated in Table 2.

The in vivo TE = **TD₅₀**/**AAM**. Confidence interval of TE estimate is (TE/1.8) ± (TE x 1.8).

Polargraphic Measurement of 1-Nitroacridine Reduction. Half-wave reduction potentials (**E₁/₂**) were measured versus standard calomel electrode with a polargraphic analyzer with a dropping mercury electrode and a standard calomel electrode. The 1-nitroacridines were dissolved in phosphate-buffered saline (in μg/ml: CaCl₂, 100; KCl, 200; KH₂PO₄, 200; MgSO₄, 59.2; NaCl, 800; NaH₂PO₄, 1150) and the solution was supplemented with 1% (w/v) gelatin (Difco Laboratories, Detroit, MI). Before polargraphic measurements, oxygen was removed from the 1-nitroacridine solutions by passing hydrogen produced electrochemically through them.

Statistical Evaluation. Associations among quantitative variables describing properties of 1-nitroacridine derivatives (Tables 2 and 3) were evaluated by means of the nonparametric Spearman's rank correlation on the basis of the S statistics (16), and by parametric analyses using the Pearson product-moment correlation coefficient (r) and significance (γ) (16).

RESULTS

The 1-nitroacridines with various modifications in the aliphatic side-chain moiety (Table 1) have proved the most cytotoxic agents among the synthesized acridine derivatives; the concentrations of 1-nitroacridines required to inhibit 72-hr growth of monolayer HeLa S₂ and HeLa cell cultures (the in vitro **ED₅₀** values) ranged from 0.0005 to 0.074 μM, and from 0.001 to 0.32 μM, respectively (Table 2). The cultured HeLa S₂ cells were generally more sensitive to inhibition by particular 1-nitroacridine derivatives than were the HeLa cells (Table 2); the shape of the curves describing the effects of increasing 1-nitroacridine concentrations on the growth of either HeLa cell line were similar for all derivatives examined (not shown).

Cessation of HeLa S₂ cell growth caused by 1-nitroacridines appears to be an irreversible process, as revealed by the cytotoxicity assays (Chart 1) in which the routine procedure (cf. "Materials and Methods") was modified; the cells were exposed to 1-nitroacridines as before but for 24 hr, washed free of the agent, and reintroduced into normal growth medium to subsequently divide and maintain themselves in the absence of the.
Biological activity of 1-nitroacridines

<table>
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<th>HeLa (EMEM, 72 hr)</th>
<th>HeLa S3 (JMEM, 72 hr)</th>
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<th>TD50</th>
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- The structural formulae and designations of 1-nitroacridines are given in Table 1.
- EMEM, Eagle’s minimal essential medium; JMEM, Joklik’s modified essential medium; ND, not determined.
- The precision of method of determination was ±0.5 to 0.7 µmol/kg (cf. also Ref. 8).
- Mean values; S.E. was <15%.
- Experimental values are those in Ref. 8.

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**Chart 1.** Correlation for 1-nitroacridines between their in vitro cytotoxic activity, $ED_{50} (\mu M)$, against cultured (Joklik’s modified essential medium, at 37°C) HeLa S3 cells after 72-hr (variable y) and 24-hr (variable x) exposure of the cells to the agents. The 24-hr incubation with the drugs was followed by replacing the culture medium with acridine-free medium; see text. The data points (n = 10) fit a straight line according to the equation $y = -0.195 + 0.957x$, with a linear regression coefficient $r = 0.993$ ($y = 0.995$).

**Chart 2.** Correlation for 1-nitroacridines between their in vivo antitumor activity, $AA_{50} (\mu M/kg)$; cf. the data in Table 2), and their in vitro cytotoxic activity (37°C; 72 hr; Eagle’s minimal essential medium) against cultured HeLa line cells, $ED_{50} (\mu M);$ cf. the data in Table 2). The data points (n = 17) fit a straight line according to the equation $log (AA_{50}) = 2.088 + 0.534 log (ED_{50})$ with a linear regression coefficient $r = 0.706$ ($y = 0.995$), and nonparametric Spearman’s rank correlation (16) indicates a significant correlation ($y = 0.95$) with $r_s = 0.520$ and $S_{50} = 392$. The data points relate the assigned 1-nitroacridine code number (cf. Table 1) to the experimental values listed in Table 2. Sa 180, Sarcoma 180.
The in vivo toxicity of 1-nitroacridines (the TD50 values listed in Table 2) is positively correlated with the in vitro cytotoxic activity of the agents (the ED50 values listed in Table 2) against either HeLa cells (parametric analysis: \( r = 0.696; \) nonparametric Spearman's rank correlation: \( r_s = 0.750; n = 16; \gamma = 0.95 \)); the latter correlations are statistically significant. The above results and considerations indicate that both the in vivo antitumor effectiveness of 1-nitroacridines and toxicity produced by these agents in vivo and in vitro are associated with their similar critical interference with cellular metabolism.

The belief that interstrand DNA cross-linking (9, 10, 12) might be responsible for the in vivo antitumor and in vitro cytotoxic effects was the basis for our interest in such 1-nitroacridine-DNA interactions. The 1-nitroacridine derivatives examined have proved without exception very effective cross-linking agents toward HeLa S3 DNA; as much as 30 to 50% cross-linked DNA molecules could be detected after 24-hr exposure of the cells to the agents (Chart 4). With increasing concentration of an agent, the proportion of cross-linked DNA molecules is increased, and the least-squares regression analysis evidences that the percentage of cross-linked DNA molecules (variable y) and the 1-nitroacridine concentration (c, the variable x) for the equation

\[
y = b_0 + b_1 \log x
\]

where \( b_0 \) and \( b_1 \) are the regression coefficients (cf. Chart 4). It is seen here that the most cytotoxic derivatives (cf. the ED50 values in Table 2) are the most effective DNA cross-linking agents (Chart 4), because the increasing 1-nitroacridine code number corresponds to less pronounced inhibitory effects toward the growth of either HeLa cell line.

A convenient way of summarizing and comparing results from a large number of phase system assays for HeLa S3 DNA cross-linked by different 1-nitroacridines was to calculate, from each plot of Chart 4 fitting Equation B, the 1-nitroacridine concentration cross-linking a given percentage of HeLa S3 DNA molecules (Table 3, Columns 2 and 3). Here, the extrapolated intercept \( c_{0} \) at 0% of cross-linked DNA is a measure of the 1-nitroacridine cross-linking potency in vivo, i.e., the \( c_{0} \) corresponds to the threshold 1-nitroacridine concentration beyond which the first chemically cross-linked HeLa S3 DNA molecules could be detected under the experimental conditions of the present study. The \( c_{0} \) values listed in Column 3 of Table 3 correspond to 1-nitroacridine concentrations introducing cross-links into 25% of DNA molecules. The \( c_{0} \) values appear to be a better measure of cross-linking capability of 1-nitroacridines than do the \( c_{25} \) values, since in the latter case, our description of DNA cross-linking by 1-nitroacridines may be complicated by the factors such as, e.g.,

![Chart 3. Correlation for 1-nitroacridines between their in vivo antitumor activity, A[AAso](µmol/kg; cf. the data in Table 2), and their in vitro cytotoxic activity, log(ED50/µM)](chart3.png)

![Chart 4. Fraction of enzymatically activated 1-nitroacridine-induced interstrand cross-linked DNA molecules after incubation (37°; 24 hr; Joklik's modified essential medium) of monolayer HeLa S3 cells with 1-nitroacridines, dependence on the 1-nitroacridine concentration, c. The data points fit a straight line according to the equation \( y = b_0 + b_1 \log x \), where the percentage of chemically cross-linked DNA molecules is variable y and the 1-nitroacridine concentration, c (µM), is the variable x; \( b_0 \) and \( b_1 \) are the regression coefficients (cf. Chart 4). It is seen here that the most cytotoxic derivatives (cf. the ED50 values in Table 2) are the most effective DNA cross-linking agents (Chart 4), because the increasing 1-nitroacridine code number corresponds to less pronounced inhibitory effects toward the growth of either HeLa cell line. A convenient way of summarizing and comparing results from a large number of phase system assays for HeLa S3 DNA cross-linked by different 1-nitroacridines was to calculate, from each plot of Chart 4 fitting Equation B, the 1-nitroacridine concentration cross-linking a given percentage of HeLa S3 DNA molecules (Table 3, Columns 2 and 3). Here, the extrapolated intercept \( c_{0} \) at 0% of cross-linked DNA is a measure of the 1-nitroacridine cross-linking potency in vivo, i.e., the \( c_{0} \) corresponds to the threshold 1-nitroacridine concentration beyond which the first chemically cross-linked HeLa S3 DNA molecules could be detected under the experimental conditions of the present study. The \( c_{0} \) values listed in Column 3 of Table 3 correspond to 1-nitroacridine concentrations introducing cross-links into 25% of DNA molecules. The \( c_{0} \) values appear to be a better measure of cross-linking capability of 1-nitroacridines than do the \( c_{25} \) values, since in the latter case, our description of DNA cross-linking by 1-nitroacridines may be complicated by the factors such as, e.g.,

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The in vitro HeLa S3 DNA cross-linking potency of 1-nitroacridines was determined after exposure (37°; 24 hr; Joklik’s modified essential medium) of cultured monolayer HeLa S3 cells to the 1-nitroacridines. The data points (n = 10) fit a straight line according to the equation log (ED50) = -2.341 + 1.190 log (c0) with a linear regression coefficient r = 0.733 (y = 0.975), and nonparametric Spearman’s rank correlation (16) indicates a significant correlation (γ = 0.95) with r = 0.636 and S = 60. The data points relate the assigned 1-nitroacridine code numbers (cf. Table 1) to the experimental values listed in Tables 2 and 3.

The in vitro DNA cross-linking potency of 1-nitroacridines, as assayed with HeLa S3 cells, correlates also with the in vivo antitumor activity of these agents against Sarcoma 180 tumor cells, as indicated by the results of either least-squares regression analysis (p < 0.025) or Spearman’s rank correlation test (p = 0.06), that verified association between the log AA50 and log c0 values (Chart 6).

The in vitro HeLa S3 DNA cross-linking potency of 1-nitroacridines correlates as well with their in vivo toxicity in the Sarcoma 180 tumor test (least-squares regression test: r = 0.740; Spearman’s test: r = 0.567; n = 9; γ = 0.95), supporting the view that the interstrand DNA cross-linking potency is very likely to be one of primary determinants of both in vivo antitumor activity and toxicity of 1-nitroacridines.

On the contrary, there is no functional relationship between any of the above in vivo and in vitro biological activities of 1-nitroacridines and their in vitro susceptibility to anaerobic reduction. Thus, except for Compound 7 which is reduced polarographically in 4 waves, the 1-nitroacridines are reduced in 5 waves, the latter 2 of which, designated as Waves 4 and 5 (Table 3) can be ascribed to reduction of acridine system (2). The first 3 waves (Waves 1, 2, and 3; Table 3) correspond to a step-wise reduction of the nitro group of the agents. The half-wave potentials of these waves (polarograms not shown) are compiled in Table 3. Statistical evaluation of these half-wave potentials indicates (the results of statistical analyses not shown) differences in repair processes,5 stability of DNA cross-links (12), repair inhibition,6 and alteration of cell metabolism (12). It is seen (Table 3) that the c0 concentrations of 1-nitroacridines are 39 (Compound 12) + 2380 (Compound 2)-fold higher than are the respective cell-growth inhibitory concentrations portrayed by the ED50 values (compare also the results for the Compound 8, Ledakrin in Ref. 12).

Least-squares regression analysis of the results with the in vivo assays with cultured HeLa S3 cells emphasizes (Chart 5) that a highly significant (p < 0.001) correlation exists between the in vitro interstrand cross-linking potency (log c0) of 1-nitroacridines and toxicity of 1-nitroacridines.

The in vitro cross-linking efficacy (µM) of 1-nitroacridines in their induction of interstrand cross-linking of HeLa S3 DNA after their incubation (37°; 24 hr; Joklik’s modified essential medium) with monolayer HeLa S3 cells, as determined by their c0 and c25 concentrations

The c0 values correspond to the threshold 1-nitroacridine concentrations beyond which the first chemically induced cross-linked HeLa S3 DNA molecules could be detected, and the c0 values are the 1-nitroacridine concentrations introducing cross-links into 25% of the DNA molecules; the values of c0 and c25 were obtained from the data presented in Chart 4. The polarographic half-wave potentials, E1/2, are ascribed to the reduction of 1-nitroacridines under anaerobic conditions; the superscripts assign the E1/2 values to the particular waves of 1-nitroacridine reduction (cf. also Ref. 2 for discussion).
that there are no significant differences in the ease of \textit{in vitro} reduction under anaerobic conditions among the 1-nitroacridine derivatives of disparate both \textit{in vivo} and \textit{in vitro} biological activity.

**DISCUSSION**

This study has proved that \textit{in vivo} irreversible interstrand cross-linking of cellular DNA is a general property of 1-nitroacridines. Until now, any claim that irreversible DNA cross-linking by 1-nitroacridines \textit{in vivo} (4, 10, 12) or \textit{in vitro} (12) might be a significant biochemical action of those agents could be properly regarded with skepticism. That skepticism was based in large part on the facts that interstrand DNA cross-linking had only been observed with the structurally related derivatives (12) which exhibited similar and very potent \textit{in vitro} cytotoxic activity (19), and that less cytotoxic isomeric nitroacridines (19) failed to induce discernible interstrand cross-linking of DNA of cultured tumor (12) or bacterial (12) cells, and of experimental animal tumor systems \textit{in vivo} (4).

A possible way of explaining this apparent difference in DNA cross-linking capability of isomeric nitroacridines was to theorize that cross-linking required accumulation of a critical concentration of the nitroacridine or its metabolite in the cell (12), and that this concentration was ordinarily not achieved with less cytotoxic nitroacridines, because their concentrations required to induce the effect (12) were too high relative to the DNA cross-linking-inhibitory side effects which were then observed (12). That theory is now of limited importance at best, because the present study shows that the 1-nitroacridines with various structural modifications (Table 1), and exhibiting wide disparity both in their \textit{in vivo} antitumor activity and toxicity and \textit{in vitro} cytotoxicity (Table 2), are all capable of effective \textit{in vitro} interstrand DNA cross-linking (Chart 4).

The \textit{in vitro} interstrand DNA cross-linking potency of 1-nitroacridines is well differentiated (Table 3). Induction of renaturable fraction in HeLa S3 DNA by some of the compounds (e.g., Codes 14 and 16) requires the concentration about 20-fold higher than those effective in the case of more potent cross-linking agents (e.g., Codes 1 and 6). The possible source of these differences could reside in unequal susceptibility of 1-nitroacridine derivatives to metabolic activation (17), the process which appears to be an essential prerequisite for the monofunctional (9, 10, 18) and/or bifunctional (10, 12) covalent binding of 1-nitroacridines with target macromolecules \textit{in vivo} (4, 9, 10, 12, 18) and \textit{in vitro} (17, 18).

One theory might be that the differences in biological action and potency among 1-nitroacridine derivatives are determined by their susceptibility to metabolic reduction of the nitro group (17), due to structural modifications in the aliphatic side-chain moiety. The finding that the crystal and molecular structure of nitroacridine antineoplastic drug named Ledakrin or Nitracrine (Code 8), as solved by X-ray analysis, is atypical as compared with acridine derivatives which include the 2-nitro analogue of this drug (for review see Ref. 6) suggested the importance of the metabolic transformation(s) of the nitro group (17) for biological potency of the nitroacridines. To provide some insight into the mechanism by which some acridine derivatives impose chemically stable bonds in cellular DNA, thereby preventing strand separation, we studied the susceptibility of 1-nitroacridines to polarographic reduction under anaerobic conditions (Table 3).

Although the complexity of the reduction of nitroheterocycles detracts from the use of polarographic half-wave potentials in the direct comparison of the reduction of different types of nitro compounds, in limited comparison, such as relative measurement of the ease of reduction among a group of structurally related (Table 1) nitroacridines, they may have application (15, 22). The uniformity of the respective \( E_{1/2} \) values (Table 3) of different 1-nitroacridines indicates that nitroreduction of the parent agents cannot be one of primary determinants of the disparate DNA cross-linking potency of the agents portrayed in Charts 3 and 4.

Obviously, the biological activity of various 1-nitroacridines with similar electron affinity may be affected by the other chemical and physicochemical factors. Within a group of compounds of the same biochemical mechanism of action, the quantitative structure-activity relationship analysis has been proven as an effective method of optimization of drug design and selection, the latter including acridine derivatives (1). Syntheses of some of the 1-nitroacridine derivatives used in this study, that is those of, e.g., Compounds 9 and 11, with the best therapeutic effectiveness indices (Table 2), was also directed by the results of the quantitative structure-activity relationship analysis (13). There are many stages of drug action, where factors like electron density, partition coefficient, and \( pK_a \) values may play an important role in affecting the biological activity of 1-nitroacridines (13). These include stability of the compound in physiological condition (6), tissue distribution (4, 6) and penetration through cell membrane (6), association with and activation by cellular enzymes (17), transfer of active metabolites to the site of action (6, 17), and interaction of the transformed products (12, 17) with cellular targets (4, 6, 12, 17-19). The current working hypothesis, toward elucidation of a unique role of a location of the nitro group in position 1 of acridine nucleus for cytotoxic and antitumor activity of the agents (6, 9, 11), is that the biological potency of 1-nitroacridines is determined by their oxidative transformations (17), the processes which are the most likely to be decisive in the total (i.e., monob- and bifunctional) binding of acridine metabolites with tissue macromolecules \textit{in vitro} (17). An alternative explanation that the DNA repair phenomena are responsible for different renaturability of DNA extracted from the 1-nitroacridine-treated cells cannot be excluded at present, although such a high specificity of DNA-repair processes does not appear very likely.

The main findings from the present study are, therefore, that the \textit{in vivo} antitumor activity and toxicity of 1-nitroacridines with various modifications in aliphatic side-chain moiety correlates with their \textit{in vitro} cytotoxic activity and their \textit{in vitro} intracellular DNA cross-linking potency. Assuming that the existence of a positive correlation might be a reflection of a causal relationship between the considered events, we propose that the irreversible (covalent) interstrand DNA cross-linking is the common biochemical mechanism of action of 1-nitroacridines, and the one of primary determinants of their both \textit{in vitro} cytotoxicity and \textit{in vivo} antitumor potency and toxic action. Our suggestion that both antitumor and toxic effects of 1-nitroacridine action \textit{in vivo} are exerted by the same biochemical mechanism does not imply in any way that poor selective toxicity of these agents is an inevitable consequence of this fact. On the contrary, we think that the unusual, multidirectional activating metabolism of 1-nitroacridines (17, 18) provides a good basis for improvement of their remarkable antineoplastic potential, the latter along with
testing of 1-nitroacridines against experimental animal tumor systems with high levels of the activating enzymes (17). The accumulation of active form(s) at the sites of action is amenable to control by a variety of structural, physicochemical, and physiological factors (21). It seems therefore possible to change favorably at least some of them through rational design and selection (21) of new compounds of the 1-nitroacridine series, and/or through a sensible application of the existing drugs.

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Cytotoxic and Antitumor Activity of 1-Nitroacridines as an Aftereffect of Their Interstrand DNA Cross-Linking

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