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Biochemical Pharmacology and DNA-Drug Interactions by CI-958, a New Antitumor Intercalator Derived from a Series of Substituted 2H-[1]Benzothiopyrano[4,3,2-*cd*]indazoles

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SUMMARY

CI-958, PD 121373, and PD 114595 belong to a new class of DNA complexers, substituted 2H-[1]benzothiopyrano[4,3,2-*cd*] indazoles, and are being further developed as antitumor drugs based on their curative properties against murine solid tumor models. The biochemical effects of these drugs on L1210 leukemia cells and their interaction with DNA were studied and compared to clinically used intercalators. The benzothiopyranoindazoles bound to DNA with a relatively high affinity, having intrinsic association constants of between 3 and 4 × 10^5 M^{-1}. Based on viscosity measurements, the mode of DNA binding appears to be through intercalation. Unwinding angles were calculated to be approximately 18°. The benzothiopyranoindazoles were potent inhibitors of nucleic acid synthesis, reducing both DNA and RNA synthesis to the same extent at similar concentrations. Like other known intercalators, these compounds produced DNA single- and double-strand breaks in a time- and concentration-dependent manner in L1210 cells. Between one and two DNA strand breaks were formed per protein-strand crosslink. Repair of these DNA lesions after the drug was removed from the cells was either very slow or did not occur at all for at least 2 hr. Finally, since the high incidence of cardiotoxicity associated with the administration of anthracyclines has been related to the formation of reactive oxygen species, the ability of the benzothiopyranoindazoles to augment superoxide dismutase-sensitive oxygen consumption was observed in a rat liver microsomal system. These compounds produced less than 5% of the activity in this assay that doxorubicin produced.

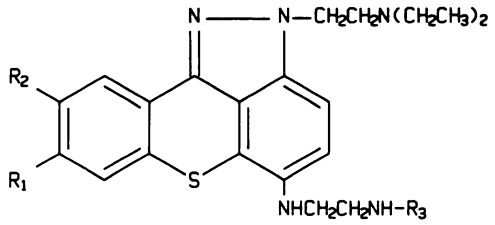
Intercalating agents continue to be among the more widely used and therapeutically active cancer agents in clinical use. The anthracyclines are currently the most prevalent in this respect, having been incorporated into a number of clinical protocols (1–3). This structural class, however, produces an accumulative and irreversible cardiomyopathy which severely limits dosage (1) and, consequently, there exists a continual search for other analogs or structures possessing the inherent properties needed to alleviate these problems. Despite the large number of chemical modifications that have been made within the anthracycline class, no analog yet has demonstrated therapeutic efficacy equal to doxorubicin, while at the same time showing a reduced tendency to cause cardiotoxicity (1, 3). As a result, a number of new structures have emerged which show potentially useful antitumor activity and possess biochemical and pharmacologic properties somewhat different from those of the anthracyclines. Among these are amsacrine (4, 5) and a second generation aminoacridine, CI-921 (6, 7), the anthracenedione, mitoxantrone (8, 9), and a recently described class of DNA binders, the anthrapyrazoles (10, 11).

The present paper describes the biochemical properties of a new class of intercalators, substituted 2H-[1]benzothiopyrano[4,3,2-*cd*]indazoles, which have shown excellent antitumor activity against a broad spectrum of tumors *in vitro* and *in vivo* (12–14). This series differs structurally from the anthrapyrazoles (10, 11) in that the carbonyl at the 6-position has been replaced with a sulfide linkage. As will be discussed, this modification confers certain biochemical characteristics which are distinctly different from previously described structural classes. This paper will describe the interaction of these compounds with DNA, their ability to cause DNA strand scission, effects on nucleic acid synthesis and production of superoxide radical. Emphasis will be placed on the three derivatives which have the broadest spectrum of antitumor activity within this series, CI-958 which has been selected for clinical evaluation, PD 114595, and PD 121373 (Fig. 1). Data for other intercalators will be included for comparison.

Materials and Methods

Chemicals. Native calf thymus DNA, ethidium bromide, superoxide dismutase, chloramphenicol, tetracycline, sodium dodecyl sulfate, NADPH, and doxorubicin were purchased from Sigma Chemical Co.

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SSB, single-strand break; DSB, double-strand break; PSC, protein-strand crosslink.



PD#	R_1	R_2	R_3
CI-958	OH	H	H
121373	H	OH	H
114595	H	OH	CH_2CH_2OH

Fig. 1. Molecular structures for CI-958, PD 121373, and PD 114595.

(St. Louis, MO). Cesium chloride was from United States Biochemical Corp. (Cleveland, OH). Tetrapropylammonium hydroxide was from Eastman Kodak Co. (Rochester, NY). [*methyl*-^{14}C]Thymidine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine were purchased from Amersham (Arlington Heights, IL). PD 114595, CI-958, and PD 121373 were synthesized and kindly supplied by Dr. Hollis Showalter (Warner-Lambert Co., Ann Arbor, MI).

Cell culture. All experiments employed L1210 mouse leukemia cells grown as a suspension culture in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin. During all drug treatments cells were in early log growth at a density of approximately 2×10^5/ml. The doubling time was 12–13 hr and viability was greater than 95% by trypan blue exclusion.

Propagation and isolation of pBR322 plasmid DNA. *Escherichia coli* K-12/pBR322 was kindly supplied by Dr. N. Waleh, Stanford Research Institute (Stanford, CA). Three liters of *E. coli* were grown in LB media containing 10 g of bacto-tryptone, 5 g of Bacto-yeast (Difco Laboratories, Detroit, MI), and 10 g NaCl/liter of water adjusted to pH 7.5 to which tetracycline was added at a concentration of 15 μg/ml. Cells were grown to an optical density of 0.5–0.6 at 600 nm, and chloramphenicol (170 μg/ml) was added and incubation continued for 14 hr. The cells were harvested by centrifugation, and the plasmid DNA was isolated and purified by established methods (15).

DNA binding studies by spectrophotometric titration. Measurements were made in a 3-ml, 1-cm length light path quartz cuvette using a Cary model 219 scanning spectrophotometer equipped with a Haake model FE2 water bath thermostated at 25°. Drugs were dissolved in 100 mM NaCl, 25 mM HEPES buffer, and 1 mM EDTA at a concentration of 50 μM, and the visible absorption spectrum (300–600 nm) was determined for each ligand in the presence of varying concentrations of calf thymus DNA. Drug solutions were titrated by the addition of small aliquots of DNA and the extinction was calculated after each addition at the absorbance maximum. The extinction of the bound drug was determined by extrapolation to infinite DNA concentration by the method of Bontemps and Fredericq (16). The absorbance of both free and bound drug was determined over the applied concentration range (0–50 μM) to ensure that the drug solutions obeyed Beer's law. The fraction of drug bound to DNA (c) after each addition during the titration was calculated from the equation:

$$c = (E_f - E)/(E_f - E_b) \quad (1)$$

where E_f is the molar extinction of the free drug, E_b is the molar extinction of the bound drug, and E is the observed extinction. From the values of c, the variables C_f (molar concentration of free drug) and r, the binding ratio (drug bound per lattice unit) were calculated, and binding parameters were estimated using the model of McGhee and Von Hippel (17) which takes into account the effects of neighboring site exclusion in the binding of ligands to a one-dimensional homogeneous lattice. In this case one lattice unit has been chosen to represent one base pair. The data were fitted to the equation:

$$r/c_f = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \quad (2)$$

where K is the intrinsic association constant and n is the binding site size (base pairs occluded by one bound ligand). Values of K and n giving best fit to the data were determined by incorporating Eq. 2 into an iterative least squares computer program designed to solve problems in which the parameters appear nonlinearly (NLLSQ, CET Research Group, LtD, Norman, OK).

Viscometry. The extent of DNA unwinding that occurred as a result of bound drug was determined by measuring changes in the viscosity of supercoiled covalently closed circular DNA (pBR322) in the presence of known concentrations of drug by a modified method of Revert *et al.* (18). Measurements were made in an Ostwald viscometer having a 10-cm capillary of 0.4 mm bore with a 0.7-ml bulb and thermostated in a glass-sided water bath at 25°. All drug and DNA solutions were made in 100 mM NaCl, 25 mM HEPES, and 1 mM EDTA at pH 7.0. The flow times for water and buffer were 76.9 and 78.5 sec, respectively. For routine experiments, 1 ml of DNA at 95 μg/ml and having a flow time of 82.4 sec was placed in the descending limb of the viscometer. Drugs were added in 10-μl increments with an Eppendorf micropipette fitted with a piece of intramedic polyethylene tubing (i.d. = 0.34 inch, o.d. = 0.05 inch) over the tip. Thorough mixing was accomplished by gently bubbling the solution with an air-filled syringe attached to the ascending limb. Flow times were made in triplicate with average deviations of no more than 0.2 sec. Reduced viscosities were calculated using established equations (19). The superhelix density for the pBR322 used in these experiments was calculated from viscometric titrations with ethidium using the equation:

$$\sigma = (\varphi_e/18)r' \quad (3)$$

where σ is the superhelix density, r' is the binding ratio at the concentration of drug producing maximum viscosity, and φ_e is the unwinding angle of ethidium, taken to be 26°. The unwinding angles (φ) for the experimental drugs were calculated from the equation:

$$\varphi = \sigma\ (18)/r' \quad (4)$$

Incorporation of radioactive precursors into macromolecules. Incorporation of radiolabeled precursors into DNA and RNA was monitored by exposing logarithmically growing cells to either [*methyl*-^{3}H]thymidine or [5-^{3}H]uridine, respectively, at a concentration of 1 μM and a specific activity of 1 μCi/nmol. At regular intervals the cells from a 1-ml aliquot were injected into 2 volumes of ice-cold 15% trichloroacetic acid, and the precipitate was collected on glass fiber filters. The filters were washed five times with 2-ml aliquots of ice-cold 15% trichloroacetic acid, dried, and placed in scintillation vials along with 10 ml of Ready-Solv (Beckman, Irvine, CA). Radioactivity was determined in a Beckman LS 6800 scintillation counter.

Oxygen consumption measurements. Microsomes were prepared from rat liver by the following method. All procedures were performed at 4°. Approximately 150 g of liver were rinsed in ice-cold 0.15 M KCl and homogenized for 1 min with a Waring blender in 3 volumes of the same solution. The homogenate was centrifuged at 15,000 × g for 15 min, the pellet was discarded, and the supernatant fraction was centrifuged for 1 hr at 100,000 × g. The pellets were resuspended in 60 ml of 0.15 M KCl plus 1 mM EDTA (pH 7.0) and centrifuged at 100,000 × g for 1 hr. Finally, the washed microsomes were resuspended in 50 ml of 50% glycerol in 0.1 M potassium phosphate, pH 7.5, at a protein concentration of 32 mg/ml and stored at −20°. The microsomes were stable for at least 1 year when stored in this manner. Protein was determined by the method of Peterson (20).

Oxygen consumption was monitored with a model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) equipped with a YSI 5331 oxygen probe. All measurements were made in a closed, water-jacketed, and continuously stirred reaction compartment at 37°. The 2-ml reaction mixture consisted of 0.2 M potassium phosphate, pH 8.0, previously aerated by bubbling with filtered air for 10 min, 1.0 mg of microsomal protein, 0.5 mM NADPH, and 5×10^{-4} M drug. Microsomes were solubilized with 2% Triton X-100 prior to measurement to

reduce endogenous activity (21). All components except drug were added to the compartment, the oxygen probe was inserted, and an endogenous rate of oxygen consumption was determined over approximately 2 min. Drug was then added via polyethylene tubing inserted in the groove of the probe, and rates were determined over a period of 2–10 min. Endogenous activity was subtracted from these rates, and data are expressed as initial rates in nmol of oxygen/min calculated from first order rate constants determined by the method of Ito and Yamamoto (22), assuming an initial concentration of dissolved oxygen of 210 μM.

DNA strand scission and DNA-protein crosslinking assays. DNA SSBs were measured by alkaline elution as described by Kohn *et al.* (23). L1210 cells in early log growth were grown in 1 μM [*methyl*-^{14}C]thymidine, at a specific activity of 0.02 μCi/nmol or 1 μM [*methyl*-^{3}H]thymidine at 0.1 μCi/nmol for 24 hr. The [^{14}C]thymidine-labeled cells were exposed to drug at a specified concentration and time interval, after which 5×10^5 cells were mixed with an equal number of [^{3}H]thymidine-labeled cells which had been previously X-irradiated with 300 rads as an internal standard. The cells were placed on polycarbonate filters (2-μm pore diameter; Nucleopore Corp., Pleasanton, CA) in a 25-mm filter holder with a 50-ml funnel (Millipore, Bedford, MA) and washed by gravity with three 5-ml aliquots of ice-cold phosphate-buffered saline. The cells were lysed by passing 5 ml of 2% sodium dodecyl sulfate/25 mM EDTA, pH 9.7, through the filter. Two ml of the same solution containing 0.5 mg of proteinase K/ml (Sigma) were layered over the cells and pumped through the filter at 20 μl/min for 1 hr, collecting the eluate into scintillation vials. The funnel was then filled with eluting buffer (tetrapropylammonium hydroxide/25 mM EDTA/0.1% sodium dodecyl sulfate, pH 12.1), and this solution was pumped through the filter at 20 μl/min with 1-hr fractions being collected for at least 16 hr. At the end of the run the filters were placed in scintillation vials and incubated with 0.4 ml of 1 N HCl at 60° for 1 hr, followed by 1 ml of 1 N NaOH for 1 hr at room temperature. Residual radioactivity in the filter holders and tubing was pumped into scintillation vials with an additional 2 ml of eluting buffer. All radioactivity was incorporated into Ready-Solv (Beckman) containing 0.7% acetic acid. Counting efficiencies were determined with ^{14}C and ^{3}H standards (Amersham) and radioactivity contributed by each isotope was determined by the method of dual channel counting. Data were graphed as a log-log plot of ^{14}C versus ^{3}H retained on the filter, and single-strand break frequency, expressed as rad eq, was calculated as previously described (23). Nuclei were isolated by the method of Filipski and Kohn (24) as described by Pommier *et al.* (25).

The frequency of DSBs was determined as described by Bradley and Kohn (26) and is similar to the procedure for SSBs except for the following. The internal standard cells were irradiated with 3000 rads and the total number of cells placed on the filter did not exceed 5×10^5. The eluting buffer was at pH 9.6.

The ratio of the frequency of true SSBs to the frequency of DSBs (*s/b*) was calculated by the method of Pommier *et al.* (25) assuming a ratio of SSBs and DSBs produced/rad of X-ray of 23.

The frequency of DNA-protein crosslinks was assessed by the method described by Zwelling *et al.* (27). Drug-treated or untreated control ^{14}C-labeled cells were X-irradiated at 4° with 3000 rads and combined with an equal number of ^{3}H-labeled cells irradiated with 300 rads. Elution methods were identical to those for single-stranded breaks except for the following. The cells were placed on 0.8-μm pore size poly(vinylchloride) acrylic copolymer (Metricell DM-800, Gelman Sciences, Ann Arbor, MI), and the lysis solution contained 2 M NaCl, 0.2% sodium dodecyl sarcosine, and 50 mM EDTA, pH 10. After cell lysis, residual lysis solution was removed by washing the filter with 3 ml of 40 mM EDTA, pH 10. Treatment with proteinase K was omitted, and DNA elution was carried out with tetrapropylammonium hydroxide/25 mM EDTA at pH 12.1. DNA-protein crosslinks were calculated using the bound-to-one-terminus model of Ross *et al.* (28).

Repair of SSBs was assessed by first incubating cells with drug for 1 hr, washing the cells twice in an excess of medium, and resuspending the cells into 10 ml of medium at 37°. Cells were then incubated at 37° and aliquots (10^6 cells) were periodically removed and lysed on filters following the procedure for SSBs.

Those procedures requiring X-irradiated cells employed a 120-kV, 5-mamp X-ray tube (Torr X-ray, Burlington, MA) set in a self-contained lead-lined cabinet (Test Equipment Distributors, Troy, MI). Cells were kept on ice during and after irradiation until they were lysed on the filters. Radiation doses were monitored with a model 500 dual polarity electrometer, equipped with a 0.33-cc probe (Victoreen Inc., Cleveland, OH).

Results

Interaction with DNA. The affinity of the benzothiopyranoindazoles for calf thymus DNA was determined by spectrophotometric titration. All compounds tested had well defined visible spectra with absorption maxima near 400 (Table 1). Upon binding to DNA the absorption maximum underwent a bathochromic and hypochromic shift with a well defined isosbestic point (Fig. 2). The extinctions for free and DNA-bound drug are shown in Table 1. A linear relationship was observed between absorbance and drug concentration over the applied concentration range for both free and bound drug, indicating compliance with Beer's law (data not shown). Fig. 3 shows Scatchard plots for the binding of CI-958, PD 121373, and PD 114595 to calf thymus DNA. The *circles* in Fig. 3 are experimental data obtained from spectrophotometric titration and the *line* is theoretical, representing the best fit to the McGhee-von Hipple equation (17). In general, there was an excellent fit between the experimental data and this equation. Table 1 shows that the three compounds tested in this series bind to calf thymus DNA with a relatively high affinity, having fairly equivalent intrinsic association constants of between 3 and 4×10^5 M^{-1}. To put these values into perspective with other known intercalators, they were about 10-fold higher than that for amsacrine and 5-fold less than that for doxorubicin (Table 1). The binding site size for this class of compounds approached 2 in all cases, which is compatible with the neighboring site exclusion hypothesis.

Evidence that these compounds bound to DNA by intercalation was obtained through effects of the drugs on the viscosity of supercoiled pBR322 plasmid DNA. Fig. 4 shows representative experiments that indicate changes in viscosity as the DNA is titrated with drug. Increasing the drug to DNA ratio resulted in the characteristic rise and fall in viscosity reflecting removal and subsequent reversal of the supercoiling. These changes are

TABLE 1

DNA binding parameters for the benzothiopyranoindazoles

All determinations were made in 100 mM NaCl, 25 mM HEPES, 1 mM EDTA at pH 7.0. The DNA used in each case was linear calf thymus DNA except for the unwinding angles, which were determined with pBR322 plasmid. Each value represents the average of three determinations.

PD No.	Parameters[a]					Unwinding angle (degrees)
	$K \times 10^{-5}$	*n*	nm	E_f	E_b	
CI-958	3.9	2.0	375	10.3	5.9	17.7
121373	3.2	1.6	389	9.1	5.5	18.5
114595	3.7	1.8	389	9.1	5.1	18.6
Doxorubicin	17.0	2.6	480	11.0	6.2	13.5
Amsacrine	0.3	2.6	434	11.5	7.4	19.0

[a] K = intrinsic association constant expressed as mol^{-1}; n = binding site size in base pairs; nm = absorption maximum and wavelength used for spectrophotometric titration; E_f = mM extinction for osmotically free drug; E_b = mM extinction for drug that is bound to calf thymus DNA.

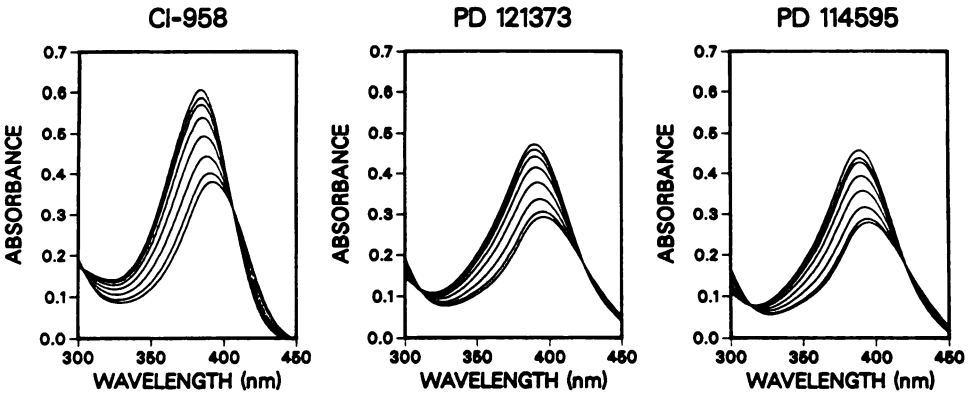


Fig. 2. Titration of the visible absorbance spectrum with calf thymus DNA. The initial concentration of drug was 50 μM in 100 mM NaCl, 25 mM HEPES, 1 mM EDTA, pH 7.0. The DNA (μM base pairs) to drug (μM) ratios from the highest absorbance maximum to the lowest, respectively, were: 0, 0.20, 0.38, 0.77, 1.37, 2.16, 3.14, and 4.12.

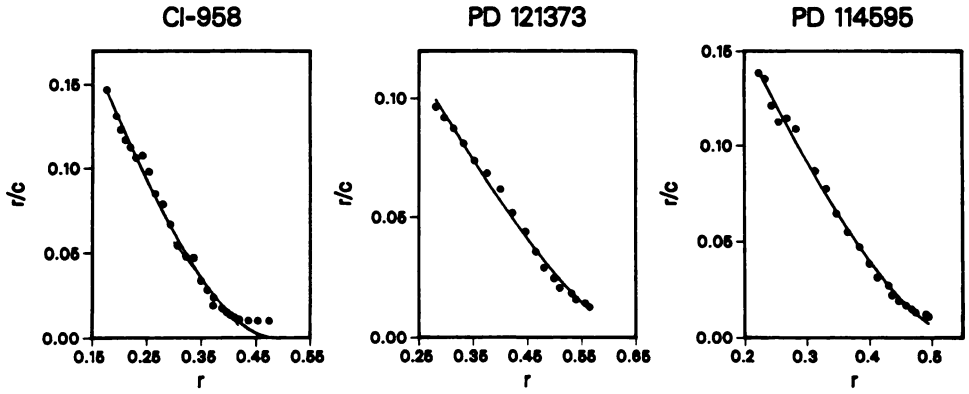


Fig. 3. Scatchard plot for the binding of the benzothiopyranoindazoles to calf thymus DNA. ●, experimental points generated from spectrophotometric titration; ——, theoretical, based on the McGhee-Von Hippel equation (Eq. 2) and representing the best fit to the data determined by nonlinear least squares analysis. r = binding ratio (drug bound/base pair); c = molar concentration of free drug.

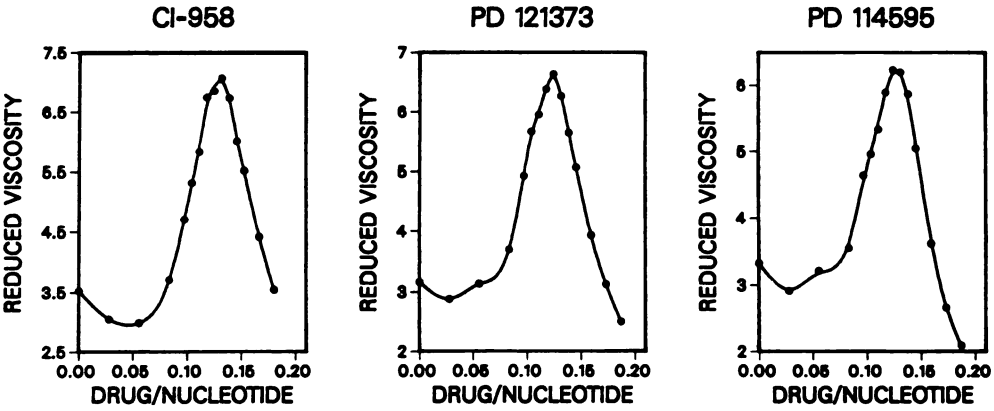


Fig. 4. Effect of the benzothiopyranoindazoles on the viscosity of pBR322 plasmid. DRUG/NUCLEOTIDE = the ratio of drug concentration to DNA concentration in bases.

most probably attributable to drug-induced unwinding of the DNA helix, which is consistent with the intercalation hypothesis. Unwinding angles were calculated to be in the vicinity of 18°.

Inhibition of nucleic acid synthesis. Table 2 shows the concentration of drug necessary to inhibit DNA or RNA synthesis by 50% in L1210 cells after a 2-hr exposure. The data indicate that the benzothiopyranoindazoles inhibit both processes to the same extent at similar concentrations. This pattern was similar to that of doxorubicin and mitoxantrone but quite distinct from that of amsacrine and the anthrapyrazoles (10) which inhibit DNA synthesis at much lower concentrations than RNA synthesis. Little difference was noted between the three analogs used in this study with each requiring less than a 1 μM concentration to inhibit by 50%.

Formation of protein-linked DNA strand breaks. DNA damage in L1210 cells exposed to the benzothiopyranoindazoles was assessed by filter elution techniques (21). Fig. 5 is a typical

TABLE 2

Effect of benzothiopyranoindazoles on DNA and RNA synthesis in L1210 cells

Cells were incubated for 2 hr at 37° with varying concentrations of drug and then exposed to tritiated thymidine or uridine for 30 min. Data are expressed as the μM concentration of drug needed to inhibit DNA or RNA synthesis by 50% in L1210 cells exposed to drug for 2 hr.

PD No./Drug	ID_{50}	
	DNA	RNA
	μM	
CI-958	0.6 ± 0.2	0.6 ± 0.1
121373	0.5 ± 0.1	0.3 ± 0.04
114595	0.5 ± 0.2	1.5 ± 0.3
Mitoxantrone	0.5 ± 0.2	0.6 ± 0.1
Doxorubicin	2.0 ± 0.5	2.1 ± 0.5
Amsacrine	0.3 ± 0.2	26.0 ± 1.0

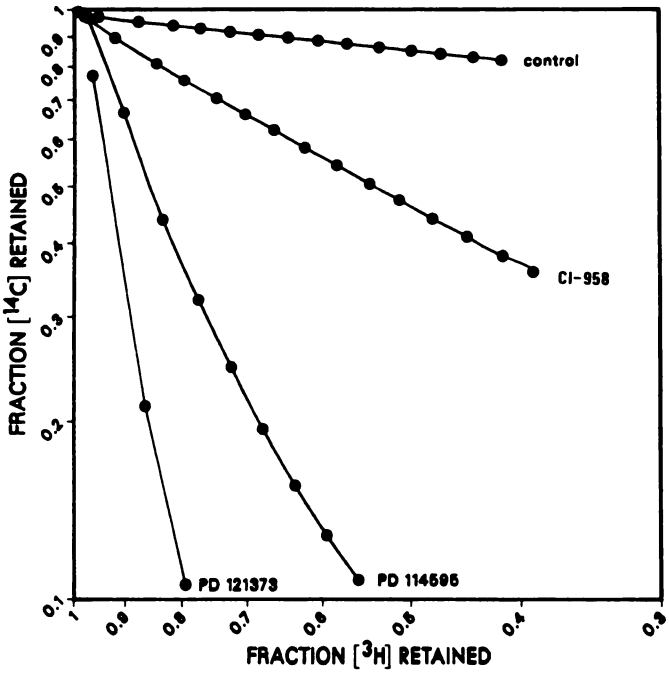


Fig. 5. Alkaline elution profile for L1210 cells exposed to 1 μM drug for 1 hr. Filter elution procedures are described under Materials and Methods.

TABLE 3

Formation of DNA SSBs and DSBs in L1210 cells exposed to 1 μM drug for 1 hr

Drug	[SSB]*	[DSB]	s/d
CI-958	450 ± 52	1284 ± 30	6.1
PD 121373	11132 ± 665	26648 ± 577	7.6
PD 114595	7765 ± 1396	9393 ± 977	17.0
Doxorubicin	186 ± 47	490 ± 40	6.7
Amsacrine	2251 ± 269	6242 ± 322	6.3
Mitoxantrone	8132 ± 1180	27680 ± 3159	4.8
Ellipticine	97 ± 28	477 ± 49	2.7

* [SSB] = single-strand DNA break frequency in rad eq; [DSB] = double-strand DNA break frequency in rad eq; *s/d* = true single- to double-strand break ratio.

alkaline elution profile in L1210 cells exposed to 1 μM drug for 1 hr and shows increased elution rates in the treated cells. Table 3 compares the relative quantity of both SSBs and DSBs generated by the benzothiopyranoindazoles and a number of control compounds and shows large differences in the number of strand breaks produced by each analog. In general, the ability of these compounds to produce DNA strand breaks correlated with potency against cells in culture (12). Table 3 also gives the true SSB/DSB ratio (*s/d*) as calculated by previously published methods (25). CI-958 and PD 121373 were similar to doxorubicin and amsacrine, with ratios between 6 and 7. PD 114595 produced a ratio 2- to 3-fold higher, perhaps due to its different upper side chain. Ellipticine produced the lowest ratio, in agreement with previous literature (29).

The association of PSCs with these strand breaks was investigated by the method described by Zwelling *et al.* (27). Table 4 shows that these drugs cause PSCs, and that the ratio of SSBs to PSCs for CI-958 and PD 121373 was near 1, similar to what has been observed for other intercalating agents (27). A ratio of 1 is consistent with what would be expected if the DNA strand scission involved topoisomerase II. The higher ratio that was observed with PD 114595 might indicate that a portion of the strand breaks is caused by another mechanism.

TABLE 4

Formation of PSCs and SSBs in the DNA of L1210 cells exposed to the benzothiopyranoindazoles

Cells were exposed to the indicated concentrations of drug for 1 hr.

Drug	Concentration	SSBs	PSCs	SSB/PSC
	μM			
CI-958	5	2862	1740	1.6
PD 121373	0.5	7340	2660	2.7
PD 114595	0.5	2396	2003	1.2

The formation of these lesions was time (Fig. 6) and concentration (Fig. 7) dependent. Figs. 6 and 7 also illustrate the marked difference in potency between CI-958 and PD 121373. One explanation for the large difference in strand-breaking capacity is differences in membrane transport rates. To investigate this possibility the quantity of strand breaks produced by these two drugs was measured in intact cells and compared with that in isolated nuclei. Table 5 shows that in intact cells, 15 times more DNA strand breaks were produced by PD 121373 than by CI-958, whereas in isolated nuclei this ratio was reduced to 2.5, indicating that slower transport of CI-958 across the plasma membrane could be a major factor contributing to the observed difference. Additional evidence to support this notion comes from a comparison of the strand breaks between cells and nuclei for each drug. Normally, a reduction in strand breaks is observed in nuclei compared to whole cells which may be due to a reduction in AT (30, 31). This pattern is observed with PD 121373; however, a 2.4-fold increase was obtained with CI-958, perhaps reflecting an increased intranuclear concentration of drug over that in whole cells.

Finally, a comparison between the rates of repair of DNA strand breaks in L1210 cells exposed for 1 hr to equally potent concentrations (those concentrations that cause equal numbers of strand breaks) of the benzothiopyranoindazoles is shown in Fig. 8. As reported previously (10, 27, 31), amsacrine-induced breaks were repaired very rapidly. Essentially no repair occurred with PD 121373 and very little with CI-958. Furthermore, additional DNA damage occurred after the drugs were removed, similar to that shown previously with doxorubicin (10). In contrast, greater than 60% of the strand breaks caused by PD 114595 were repaired within 1 hr, but, thereafter, repair appeared to cease.

Oxygen consumption studies. Table 6 shows the capacity of the benzothiopyranoindazoles to consume oxygen in the presence of rat liver microsomes and NADPH. Previous studies have shown that this activity is related to the formation of superoxide radical and subsequent production of other reactive oxygen species which may contribute to the cardiotoxic effects often experienced with intercalators (32). The benzothiopyranoindazoles produced less than 5% of the activity in this assay that doxorubicin produced, which indicates much less participation in electron recycling.

Discussion

DNA complexers have been established as among the most effective classes of antitumor agents in clinical use and are employed against a number of malignant diseases (1–3). The use of these drugs, especially the anthracyclines, has been limited, however, primarily by cardiotoxicity which ranges from a delayed and insidious cardiomyopathy to irreversible congestive heart failure (1). Considerable efforts have been made to

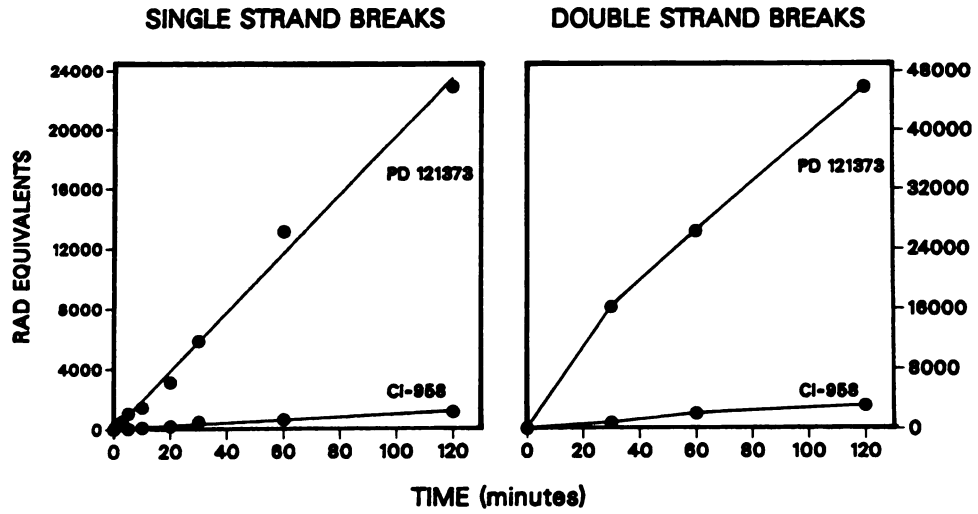


Fig. 6. Time course for the formation of DNA SSBs and DSBs (expressed as X-ray equivalents) in L1210 cells exposed to 1 μM drug.

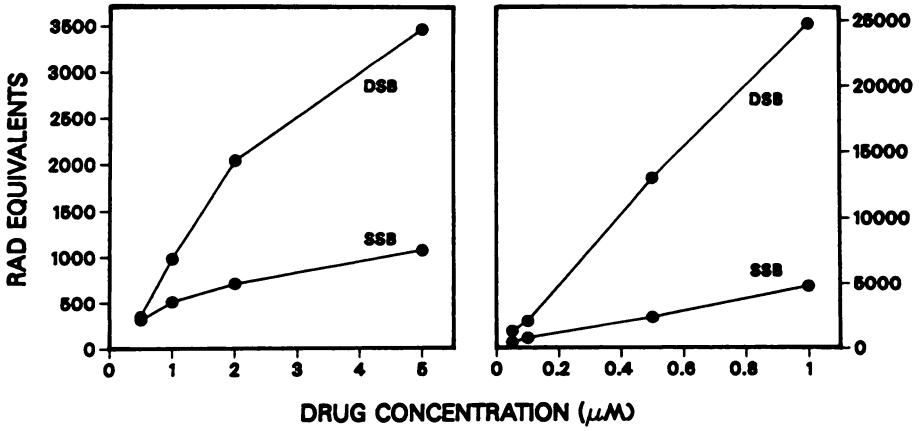


Fig. 7. Drug concentration dependence for the production of DNA SSBs (expressed as X-ray rad equivalents) in L1210 cells exposed to benzothiopyranoindazoles for 1 hr.

TABLE 5

DNA SSB frequency in L1210 cells or isolated nuclei exposed to CI-958 and 121373

Cells or nuclei were exposed to 1 μM drug for 1 hr.

	SSBs (rad eq)	
	CI-958	PD 121373
Intact cells	198	3082
Isolated nuclei	474	1173

eliminate these problems through structure modification (1, 3) as well as development of other classes of intercalating agents, such as aminoanthraquinones (8, 9), acridine derivatives (4–7), and anthrapyrazoles (10, 11). A continuing effort to develop unique intercalating agents with minimal cardiotoxicity and less potential for semiquinone radical formation has resulted in a series of 2-(aminoalkyl)-5-amino-2H[1]benzothiopyrano[4,3,2-*cd*]indazoles. This series differs structurally from a previously reported series, the anthrapyrazoles (10, 11), in that the carbonyl group at the 6-position has been replaced with a sulfide linkage. The most active compounds in this series, which are the three reported in this paper, exhibited broad spectrum antitumor activity, being curative in P388 leukemia and the MX-1 mammary human xenograft, and exhibited high levels of activity against L1210 leukemia, B16 melanoma, the Ridgway osteogenic and M5076 sarcomas, mammary adenocarcinoma 16C, and colon adenocarcinoma 11a (13, 14). The present data firmly establish that the benzothiopyranoindazoles bind to DNA with a relatively high affinity and exhibit intrinsic association constants on the order of 3–4 × 10^5 M^{-1}. Under identical conditions these values were about 5-fold less than doxorubicin and 10-fold more than amsacrine. The observation that these compounds efficiently remove and reverse the supercoiling of closed circular duplex pBR322 DNA provides strong evidence that the mechanism of this binding is via intercalation between the base pairs. The fact that the binding site size approximates 2 in every case and that the curvature in the experimental data is predicted accurately by the equation of McGhee and Von Hippel (17) suggests a binding model which allows for the effect of neighboring site exclusion. The similarity between the analogs with regard to intrinsic association constant and the unwinding angles suggest that interconversion between the 8- and 9-hydroxy substitution does not alter DNA binding, nor does modification of the lower side chain from 5[(2-aminoethyl)amino] to 5-[[2-[(2-hydroxyethyl)amino]ethyl]amino]. Although strict correlations between the affinity of intercalators for DNA and antitumor efficacy do not exist, a few studies, in which a large number of analogs within a single series have been examined, such as 9-anilinoacridines (33), do reveal a positive correlation, although factors other than gross DNA binding enter into the equation.

The benzothiopyranoindazoles were potent inhibitors of nucleic acid synthesis inhibiting both DNA and RNA synthesis

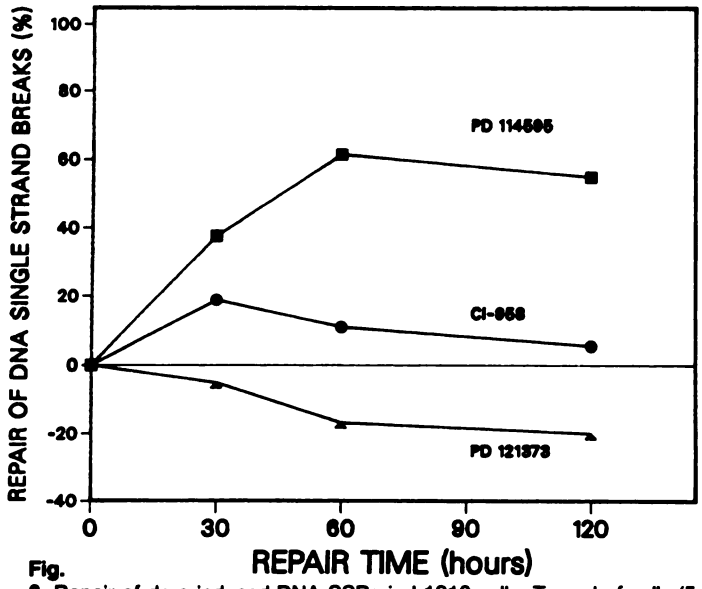


Fig. 8. Repair of drug-induced DNA SSBs in L1210 cells. Ten ml of cells (5 × 10^5) containing [^{14}C]DNA were exposed to equally potent concentrations of the indicated drugs, washed two times in 10 ml of 37° medium, and resuspended into 25 ml of 37° medium. Five ml (10^6 cells) were removed periodically and subjected to alkaline elution as described under Materials and Methods. Data are expressed as a percentage of repair. The concentrations of drug were: CI-958, 5 μM; PD 121373, 0.5 μM; and PD 114595, 1 μM.

TABLE 6

Stimulation of microsomal oxygen consumption by benzothiopyranoindazoles

Values represent nmol of oxygen consumed in the presence of 500 μM drug, 500 μM NADPH, and 1 mg of rat liver microsomal protein.

PD No./Drug	Oxygen consumption
CI-958	6.8
121373	2.1
114595	4.2
Mitoxantrone	2.0
Doxorubicin	144

to an equal extent at approximately the same drug concentration. Historically, intercalators appear to fall into three distinct categories based on either equal or preferential inhibition of DNA and/or RNA synthesis. The present compounds are similar in this respect to doxorubicin and mitoxantrone, which inhibit both processes equally. Other intercalators, exemplified by amsacrine and the anthrapyrazoles (10), inhibit DNA synthesis much more potently than RNA synthesis, whereas actinomycin D and certain anthracycline analogs (34) preferentially inhibit RNA synthesis. Since a range of DNA association constants can be found in all three groups, the discriminative or equal inhibition of DNA or RNA synthesis most likely derives from properties distinct from differences in binding affinity. Possibilities might be related to dissociation kinetics of the drug-DNA complex (35), the orientation of the bound molecule, or interaction with DNA or RNA polymerases (36–38).

The biochemical basis for the high incidence of cardiotoxicity observed with anthracyclines has received much attention in the last several years. Although still not fully understood, evidence is beginning to implicate the production of reactive oxygen species as a contributing factor, including superoxide radical, hydroxyl radical, hydrogen peroxide, and lipid peroxides (32). The capacity for many quinone anticancer agents to be reduced to semiquinone free radicals by intracellular reductive enzymes has been supported by electron spin resonance studies (39, 40). Reports showing superoxide dismutase-sensitive oxygen consumption in the presence of liver microsomal or heart sarcosomal preparations (41, 42) and the production of lipid peroxidation products (43, 44) suggest that semiquinone free radicals are capable of donating electrons to molecular oxygen, thus forming superoxide radical, or to unsaturated lipids, producing lipid peroxides. In this context, the benzothiopyranoindazoles induced far less (50- to 70-fold) superoxide dismutase-sensitive oxygen consumption than doxorubicin in the rat liver microsomal system, a property that may be indicative of a lesser cardiotoxicity.

Although the precise mechanism by which intercalators produce their cytotoxic and antitumor effects is not fully understood, strong evidence points toward production of protein-linked single- and double-strand DNA breaks as the most likely candidate (45–47). Furthermore, strong evidence indicates that the protein which is associated with and responsible for these breaks is topoisomerase II (47, 48). The benzothiopyranoindazoles are similar to other intercalators in these respects, in that they produced both SSBs and DSBs in a time- and concentration-dependent manner. These breaks were tightly associated with protein, and quantitation of both showed a ratio of 1–2 SSBs for each protein-strand crosslink, which approaches a value consistent with the currently proposed mechanism for topoisomerase II and its interaction with intercalators (49, 50). Ratios higher than 1, however, can indicate mechanisms other than those involving topoisomerases. A striking feature concerning DNA strand breakage by the benzothiopyranoindazoles is the marked rapidity of cleavage by the 9-hydroxy (PD 121373) as compared to the 8-hydroxy (CI-958) derivative. Although a number of previously studied intercalators can be classified as causing strand breaks either slowly, such as doxorubicin and ellipticine, or rapidly, like mitoxantrone and amsacrine, these compounds are derived from different structural classes and might be expected to have distinct properties. The present compounds, however, differ only in the position of a single hydroxyl and, based on the studies with isolated nuclei, a part of the difference in potency may reflect different transport rates into whole cells. The difference in the rate of strand cleavage does not seem to govern antitumor efficacy since members from both groups are active; however, based on previous *in vitro* and *in vivo* activity, it does correlate with potency (12, 13). Thus, the IC_{50} values for CI-958 and PD 121373 are 7.1×10^{-8} and 2.6×10^{-9} M, respectively (data from Ref. 14), which represents a 27-fold difference in potency. From Table 3 it can be seen that there is a 25- and a 21-fold difference in production of SSBs and DSBs, respectively. It is of interest that there exist such large differences in DNA strand breakage between these two analogs and, yet, very little difference in the inhibition of nucleic acid synthesis. This seems to indicate that the degree of inhibition of DNA and RNA synthesis is not necessarily proportional to the quantity of DNA strand breaks.

The cytotoxicity of a drug may also be related to the inability of a cell to recover from or repair the DNA breaks induced by intercalators rather than the quantity of these lesions. Strand breaks by PD 121373 or CI-958 were either not repaired or, at best, very slowly only over the first 30 min after drug removal,

and additional lesions occurred thereafter. This pattern was similar to that of doxorubicin and the anthrapyrazoles but was quite different from that of amsacrine, from which the cell recovered very quickly (10). The additional breaks occurring after drug removal may be due to drug sequestered within the cell or within membranes, as has been suggested for doxorubicin and ellipticine (51), or perhaps to DNA degradation in dead or dying cells. Approximately 60% of the DNA strand breaks caused by PD 114595 were ligated over 60 min, and then repair ceased. Fast repair of a certain percentage of strand breaks and then a slower process has been observed with mitoxantrone (10) but, as yet, it is not known whether the multiple repair rates represent more than one cell population, different sites on the chromatin, or the difference between repair of SSBs versus DSBs.

In conclusion, the benzothiopyranoindazoles are a new class of antitumor agents which bind tightly to DNA by an intercalative mode. Many of the biochemical effects are similar to those of doxorubicin in that they suppress both DNA and RNA synthesis to the same extent and cause irreversible single- and double-strand DNA breaks. Unlike doxorubicin, however, these compounds appear to have much less potential for semiquinone radical formation.

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