A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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EVALUATION OF CROSS-LINKS IN COVALENT COMPLEXES OF 1-NITROACRIDINES AND BIS-1-NITROACRIDINES WITH DNA*

An anticancer drug nitracrine (Ledakrin, C-283) and its other biologically active 1-nitroacridine analogues in the presence of dithiothreitol form with DNA irreversible complexes. The complex formation may be demonstrated by the decrease of transcriptional template activity of DNA previously incubated with a drug and sulfhydryl compounds. The aim of the present experiments is to assay the content of cross-links in the 1-nitroacridines and bis-1-nitroacridines complexes with DNA. The complexes were analyzed by means of column chromatography on hydroxylapatite following thermal denaturation. The comparison of the data concerning the biological effects of different analogues of nitracrine with the observations on thiol-dependent inhibition of RNA synthesis in vitro and the formation of covalent bifunctional complexes both in vitro and in the cell indicates that these phenomena are manifestations of the same property of the drugs.

It has been previously shown [1, 3, 4, 9] that an anticancer drug nitracrine (Ledakrin, C-283) and several of its 1-nitro-9--aminoalkylacridine derivatives in the presence of dithiothreitol exhibited a high increase of the template toxicity in DNA-dependent RNA synthesis in vitro system. The enhancement of the inhibition is due to a covalent binding of the drug to DNA. This phenomenon is observed for the nitracrine analogues which exhibit cytostatic and cytotoxic activity while it does not occur for acridines from this group with low biological activity [4, 9].

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The aim of the present experiments is to assay the content of cross-links in the nitroacridine-DNA complexes formed in vitro.

MATERIALS AND METHODS

Monomeric acridines (see Fig. 1 for their formulae) were gifts of Prof. A. Ledóchowski (Technical University of Gdańsk, Poland),

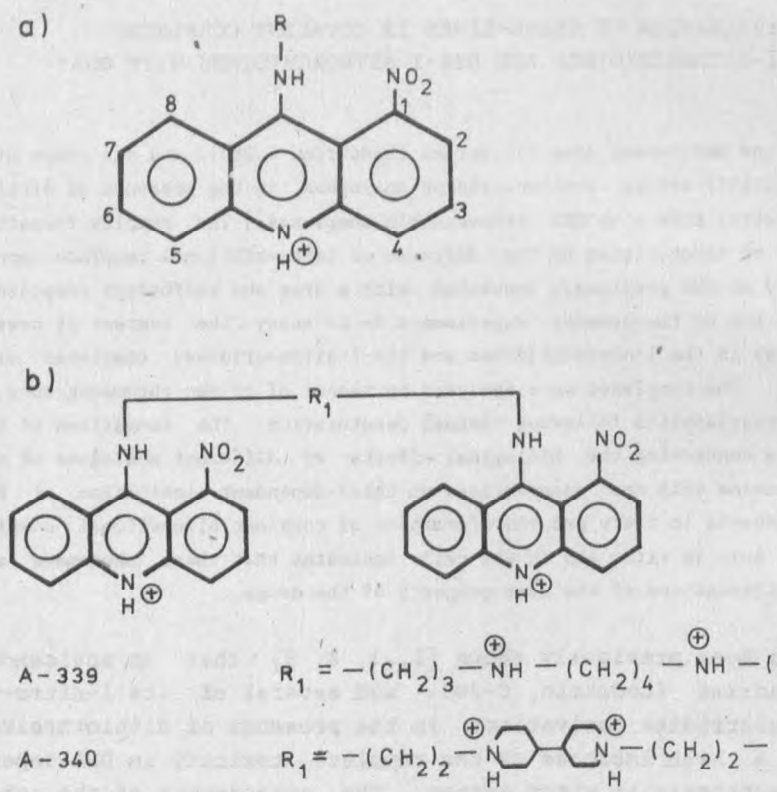

Fig. 1. Formulae of acridines studied

a) nitracrine (Ledakrin, C-283), R = $-(CH_2)_3-\overset{+}{N}H(CH_3)_2$; C-846, R = $-(CH_2)_3-\overset{+}{N}H_2CH(CH_3)_2$; C-849, R = $-(CH_2)_3\overset{+}{N}H_2CH_3$; C-857, R = $-(CH_2)_2OH$; C-921, R = $-(CH_2)_2COOH$; C-1006, R = $-(CH_2)_3\overset{+}{N}H_2(CH_2)_5CH_3$; b) A-339 and A-340

Rys. 1. Wzory badanych akrydyn

a) nitrakryna (Ledakrin, C-283), R = $-(CH_2)_3-\overset{+}{N}H(CH_3)_2$; C-846, R = $-(CH_2)_3-\overset{+}{N}H_2CH(CH_3)_2$; C-849, R = $-(CH_2)_3NH_2CH_3$; C-857, R = $-(CH_2)_2OH$; C-921, R = $-(CH_2)_2COOH$; C-1006, R = $-(CH_2)_3\overset{+}{N}H_2(CH_2)_5CH_3$; b) A-339 i A-340

Bis-1-nitroacridines (Fig. 1) were kindly donated by Dr J.-B. Le Pecq (Institut Gustave-Roussy, Villejuif, France). Escherichia coli DNA-dependet RNA polymerase was isolated as previously described [11]. Other materials used were the same as before [2, 4, 9, 11].

The complexes were formed by incubation of calf thymus DNA (400 μg/ml) with 1-nitroacridines (0.2 mmole/l) or bis-1-nitroacridines (0.09-0.1 mmole/l) in the presence of dithiothreitol (2 mmole/l) for 1 h at 0.1 mole/l of KCl and then purified by extraction with isobutanol and dialysis [2, 4, 9, 11]. The template activity of the complexes was assayed using the amount of the complex equivalent to 20 μg of DNA and [^{14}C] ATP as a labelled substrate [4, 8, 9, 11] and expressed as a percentage of RNA synthesized with the control DNA (i.e., DNA without an inhibitor). The complexes of 8-methoxypsoralen (46 μmole/l) with DNA were formed by irradiation and purified as described [2, 11].

The heat denatured complexes were subjected to chromatography on hydroxylapatite column as described before [2]. The fractions were eluted with linear gradient of 0.1-0.5 mole/l of sodium phosphate buffer pH 6.8 and absorbance of DNA was measured at 260 nm [2].

DNA concentration on the complexes were estimated spectrophotometrically [2, 11].

RESULTS

High inhibition of RNA synthesis, 8.8, 7.6, 8.6 and 10.4% of the template activity of control DNA (Tab. 1), was found with complexes of nitracrine, C-846, C-849 and C-1006 with DNA respectively. C-857 and C-921 exhibited lower inhibitory effects (32.8 and 18.3% of the control respectively). Both bis-1-nitroacridines showed also lower inhibitory effect than nitracrine used either at twofold lower or similar concentration in the dithiothreitol--dependent reaction with DNA (Tab. 1).

Covalent binding of a reactive molecule to DNA may be either monofunctional or bifunctional. In the latter case a drug may form interstrand cross-links. They may be conveniently detected after thermal denaturation by the tendency of the cross-linked DNA to reconstitute the double-stranded structure upon subsequent

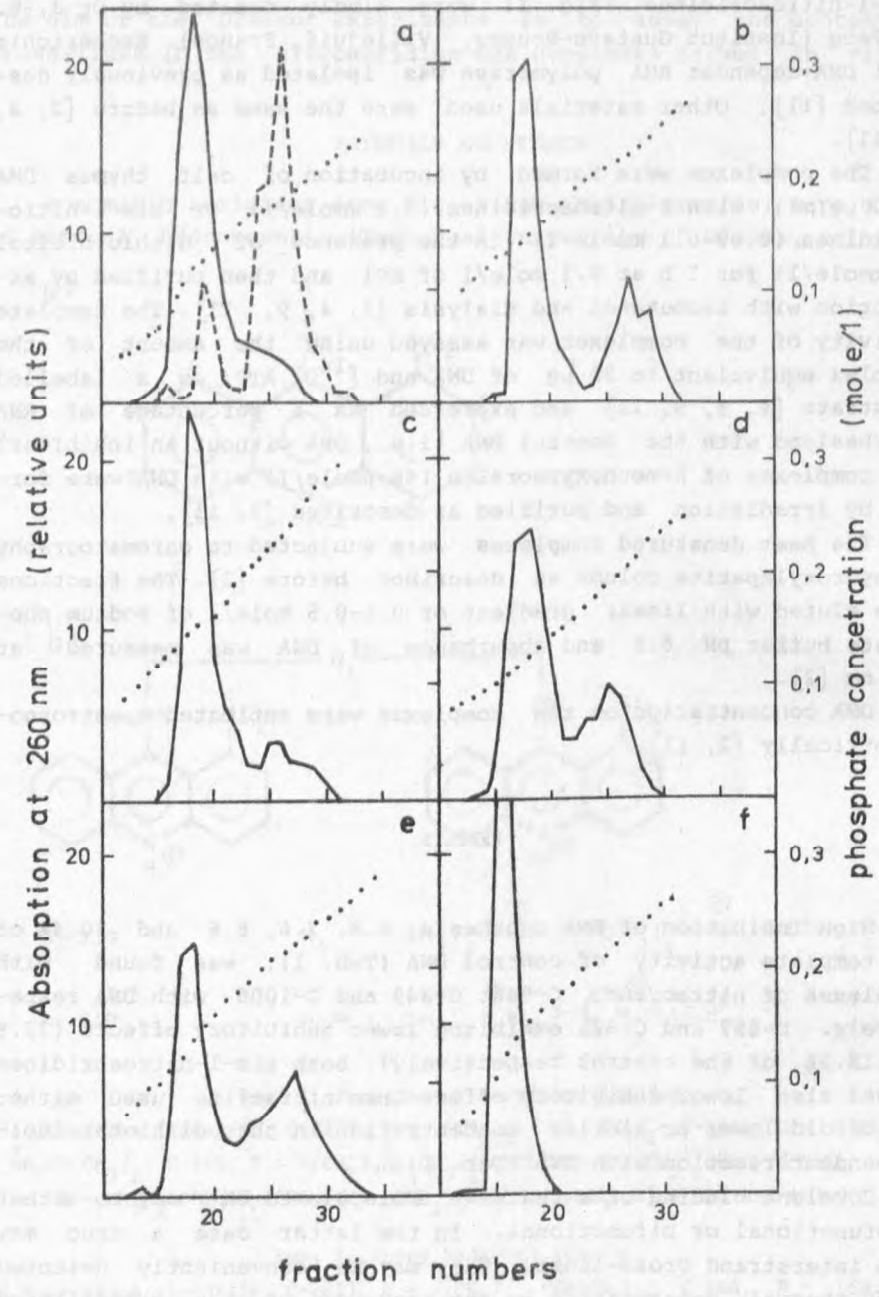

Fig. 2. Hydroksylapatite chromatography of denatured complexes of DNA with 1--nitro and bis-1-nitroacridine derivatives. Thermally denatured DNA (control DNA) and denatured complexes of DNA with 8-methoxypsoralen (a), C-846 (b),

cooling. Thermally denatured (predominantly single-stranded) and double-stranded DNA can be eluted from hydroxylapatite column at different phosphate concentrations. As already shown [2] a denatured nitracrine - DNA complex contains a small (15%, see Tab. 1) but distinct fraction of renaturated DNA.

A profile of denatured control DNA presents typically a main peak which is eluted at the phosphate concentrations of 0.14-0.15 mole/l and followed by a tail which does not from a discrete peak (Fig. 2a, solid line). Denatured complexes of DNA with 8-metoxypsoralen. an efficient cross-linking agent, were used as positive controls in our experiments [2]. Two peaks, the first corresponding to single-stranded DNA and the second one containing about 75% of the ultraviolet absorbing material were eluted from the column (Fig. 2a, dashed line). Complexes of C-846 (Fig. 2b), C-1006 (Fig. 2d) and A-339 (Fig. 2e) with DNA give two discrete peaks. A smaller shift of the ultraviolet absorbing material to the region of double-stranded DNA was observed in the elution profile of the complexes of C-857 (Fig. 2c), C-849 and C-921 (unshown) while the complex of A-340 was eluted as a single peak (Fig. 2f). The increase of the amount of DNA in the second peak was estimated by substraction of the relative absorbance of control DNA eluted in this region from the optical density of corresponding fractions of the complexes [2]. The results of several determinations are recorded in Tab. 1. The content of renatured DNA in complexes of nitracrine, C-846, C-1006 and A-339 was on the average 15, 20.5 and 21.3% while the other complexes showed lower (C-849 and C-857) if any (C-921, A-339) increase in the renaturation ability (Tab. 1).

C-857 (c), C-1006 (d), A-339 (e) and A-340 (f) were subjected to chromatography on hydroxylapatite column. Ultraviolet absorpion of each fraction is expressed as a percentage of the total absorbance recovered in the effluent

(.....) - sodium phosphate concentration (mole/l); (———) - absorbance of DNA; (------) - of 8-methoxypsoralen complex (see Fig. 2a); (———) - absorbance of nitroacridines complexes (Figs. 2b-2f)

Rys. 2. Profile zdenaturowanych kompleksów DNA z 1-nitro i bis-1-nitroakrydynami rozdzielanymi na kolumnie hydroksyapatytowej. DNA - kontrolny i DNA-8-metoksypsoralen (a), DNA - C-846 (b), DNA - C-857 (c), DNA - C-1006 (d), DNA - A-339 (e) i DNA - A-340 (f) po denaturacji poddano analizie chromatograficznej. Absorpcję (260 nm) każdej frakcji wyrażono jako procent całkowitej absorbancji materiału wypływającego z kolumny

(.....) - gradient buforu fosforanowego (mol/litr); (———) - absorpcja DNA (-----) - kompleks DNA z 8-metoksypsoralenem (patrz rys. 2a); (———) - absorpcja kompleksów nitroakrydyn (rys. 2b-2f)

T a b l e 1

Relative transcriptional template activity
and the content of cross-linked fractions
in purified complexes of DNA with 1-nitroacridines

Względna aktywność matrycowa i zawartość frakcji usieciowanej
oczyszczonych kompleksów 1-nitroakrydyn z DNA

Drug	Template activity (%)		Cross-linked fraction (%)	
nitracrine	8.8[a]		15[a]	
C-846	7.6 ± 1.4	(3)	20.5 ± 7.2	(3)
C-849	8.6 ± 2.6	(3)	7.7 ± 3.8	(2)
C-857	32.8 ± 12.8	(3)	6.8 ± 1.6	(5)
C-921	18.3 ± 3.7	(3)	0	(3)
C-1006	10.4 ± 5.4	(3)	21.8 ± 7.1	(7)
A-339	24.5 ± 1.5	(2)	19.0 ± 8.8	(3)
A-340	60.0 ± 8.0	(2)	0[b]	

[a] See ref. [2].

[b] No ultraviolet absorbing material followed the main peak, see Fig. 2f.

N o t e: Increase of the amount of DNA eluted in the region corresponding to double-stranded DNA (the cross-linked fraction) was estimated by subtraction of the absorbance (in relative units) of control DNA eluted in this region from the optical density of corresponding fractions of the complexes. Nitracrine-DNA complex formed at the drug concentration of 0.1 mmole/l had a template activity of about 13%. The number of independent experiments is indicated in the parentheses. The averages of independent experiments ± range values (for n = 2) or standard deviation values (for n > 2) are shown.

DISCUSSION

High biological activity of nitracrine and the monomeric 1-nitro-acridines assayed here was demonstrated both on cell growth in vitro and experimental tumour bearing mice (see [1, 3, 5, 7] and the literature cited therein). For example drug concentrations causing 50% inhibition of HeLa cell growth (ED_{50}) for all of the 1--nitro-acridines, except for C-921, were within the range 2-15 μmole/l, while ED_{50} for the latter was about 4 mmole/l [7]. They showed interstrand DNA cross-link formation, which was correlated with their cytotoxic and antitumour activity [7]. Unlike other acridines the biologically active 1-nitro-acridines form with DNA in the presence of thiol compounds irreversible probably covalent

complexes. As no analytically useful changes in optical properties of the complexes have been found the decrease of the template activity of DNA with E, coli RNA polymerase in standard conditions is used as a measure of the drug binding. Higher inhibition of RNA synthesis was found with the 1-nitro-9-aminoalkyl derivatives bearing the additional aminoalkyl groups in the side chain (nitracrine, C-846, C-849, and C-1006, see Fig. 1 and Tab. 1). Three of them (except C-849) form complexes of considerably, increased content of fast renaturating fraction of DNA due to the interstrand covalent binding. It is generally assumed that the 1-nitro and the additional aminoalkyl groups are two reactive sites in the molecule responsible for biological activity of 1-nitroacridines [1, 3, 5, 7]. C-857 and C-921 having hydroxyl and carbocyl groups in the side chain (Fig. 1) exhibit lower inhibitory effect and cross-linking potency (Tab. 1).

In any case however bifunctional binding of 1-nitroacridines leading to cross-link formation in DNA seems to be a relatively rare event when compared with the psoralen derivative (Fig. 2a, dashed line). The total binding of 8-methoxypsoralen determined with the tririated preparation was under these conditions about five molecules per 10^3 DNA nucleotides. An observation made with nitracrine that a log/log plot of the relative template activity versus the number of ^{14}C-labelled drug molecules bound per 10^3 DNA nucleotides presents a linear dependence [8] allows to find the approximate density of C-846, C-849 and C-1006 bindings to be about 8-15 molecules per 10^3 nucleotides. The level of the activity of DNA complexes with C-857 and C-921 is equivalent to about tenfold lower binding. Recently several dimers of nitracrine have been synthesized (L e P e c q et al. [6] unpublished experiments, see also [10]). The two bis-1-nitroacridines assayed in this paper exhibit biological effects in several tests in vivo and in vitro although their activity is lower than that of the parent compound (L e P e c q et al. [6] unpublished results). Similary to their monomers the dimers bind irreversibly to DNA in the presence of dithiothreitol, decreasing the template activity (Tab. 1). The level of binding approximated as above is about tenfol (A-339) and twentyfold (A-340) lower than that of nitracrine. As the dimers have apparently a double number of reactive sites it has been assumed that they should form cross-links in DNA upon binding. Indeed denatured complexes of DNA with A-339 are resolved on hydroxylapatite column into two peaks (Fig. 2e).

Rather surprisingly no cross-links are detected in complex of A--340 which is reproducibly eluted from the column as a single and very narrow peak (Fig. 2f). The difference between the two ligands depends on the structure of the linkers. Conformational rigidity of the diethyldipiperidine chain of A-340 (Fig. 1) may reduce the ability of both reactive sites in the drug molecule activated by dithiothreitol to interact simultaneously with DNA. No such limitation of mobility may be ascribed to a flexible spermine chain of A-339 (Fig. 1). The comparison of the data concerning the biological effects of different analogues of nitracrine with the observations on thiol-dependent inhibition of RNA synthesis in vitro, and the formation of covalent bifunctional complexes both in vitro and in the cell indicates that these phenomena are manifestations of the same property of the drugs.

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BADANIE WIĄZAŃ POPRZECZNYCH W NIEODWRACALNYCH KOMPLEKSACH NITROAKRYDYN I BIS-NITROAKRYDYN Z DNA

Lek przeciwnowotworowy Ledakrin (nitrakryna, C-283) i jego aktywne biologiczne pochodne 1-nitro w obecności ditiotreitolu tworzą z DNA nieodwracalne kompleksy. Powstawanie kompleksów można wykazać poprzez badanie obniżenia aktywności transkrypcyjnej DNA uprzednio inkubowanego z lekiem w obecności związków sulfhydrylowych. Celem pracy było zbadanie zawartości frakcji usieciowanej w kompleksach 1-nitroakrydyn oraz bis-1-nitroakrydyn z DNA. Kompleksy po denaturacji termicznej analizowano metodą chromatografii na kolumnie hydroksyapatytowej.

Porównanie danych odnoszących się do efektu biologicznego różnych analogów nitrakryny ze stopniem hamowania syntezy RNA in vitro oraz tworzeniem przez nie dwufunkcyjnych wiązań z DNA, wskazuje, że zjawiska te zależą od tych samych determinant strukturalnych badanych pochodnych nitroakrydyn.