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
FOLIA BIOCHIMICA ET BIOPHYSICA 6, 1988

Małgorzata Czyż, Kazimierz Studzian, Andrzej Wasilewski,
Aleksandra Poziomska, Dorota Wilmańska, Marek Gniazdowski

COVALENT BINDING OF NITRACRINE (LEDAKRIN, C-283) TO NUCLEIC ACIDS AND PROTEINS

In the presence of sulfhydryl compounds an anticancer drug nitracrine (NAC), 1-nitro-9-(3, 3 N, N-dimethylaminopropylamino) acridine binds irreversibly to DNA and RNA. The stability of the drug complex with DNA and RNA under various conditions were characterized. The following facts indicate the drug in the purified complexes is covalently bound to nucleic acids: i) [^{14}C] NAC in the complex is precipitated with trichloacetic acid (TCA) with similar efficiency as DNA or RNA; ii) the complex is not dissociated by 7 M urea, sodium dodecyl sulfate (SDS) or high ionic strength; iii) neither thermal nor alkaline denaturation considerably affect the efficiency of precipitation of [^{14}C] NAC-DNA with TCA. In the presence of DTT [^{14}C] NAC binds irreversibly to both albumin and histone at the level about tenfold lower than that found for DNA.

INTRODUCTION

The anticancer drug nitracrine (Ledakrin, C-283), NAC, 1-nitro-9-(3, 3-N, N-dimethylaminopropylamino) acridine undergoes metabolic activation in the cell leading to covalent binding to DNA and probably to RNA and proteins [1, 4, 8]. In the presence of sulfhydryl compounds the drug forms with DNA and RNA irreversible complexes [2, 5]. There is a correlation between the thiol-dependent binding of several nitracrine analogues to DNA and their biological effects [5, 6, 10]. The aim of these experiments is to characterize the stability of NAC bound to macromolecules.

MATERIALS

Albumin, fraction V according to Cohn and Hyamine 2389 (Serva, Heidelberg, GFR), calf thymus histon H 1 kindly donated by Dr. Zofia Milewska from our Institute, wheat germ high molecular weight RNA (Calbiochem, USA), GF/C filters (Whatman, England), Sephadex G-50 and G-100 (Pharmacia, Sweden) and calf thymus DNA (Worthington, USA) were used. 9-[^{14}C] NAC (specific activity 3-6 · 10^3 cpm/nmole) was previously described [2].

METHODS

Estimation of stoichiometry of complexes of NAC with nucleic acids and proteins. If not stated otherwise the complexes were formed by incubation of nucleic acids or proteins with NAC in the presence of 2 mM dithiothreitol (DTT) for 1 h, purified by extraction with isobutanol and their stoichiometry estimated as described before [2, 11] except that DNA and RNA concentrations were 400 µg/ml and histone (H 1) concentration 2 mg/ml. KCl or NaCl and NAC concentrations are indicated in the description of experiments. Complexes of albumin were formed as described above (albumin concentration was 2 mg/ml) but purified by filtration through a Sephadex G-50 column (1.5 x 26 cm) preciously equilibrated with 0.05 M phosphate buffer. Protein concentration was estimated according to L o w r y [7].

Assay of stability of complexes of NAC with nucleic acids. Except for sodium dodecyl sulfate (SDS) (see below) the stability of complexes of NAC with nucleic acids under different conditions was estimated as follows: 0.2 ml aliquots of the purified complex of NAC with DNA or RNA (250-400 µg/ml) were made up to 1 ml by the addition of the corresponding component (20 mM Tris/ /HCl, pH 7.5, HCl, NaOH, urea) and incubated as specified, then the samples assayed at low or high pH were neutralized. The samples were precipitated with 5 ml of 10% TCA at 0°C for 3 h, the precipitates were collected on GF/C filtres, washed 5-6 times with cold 5% TCA then once with ethanol. The filters were placed in counting vials, then 0.5 ml of Hyamine, 10% was added to each vial. The samples were incubated for 24 h at 50°C, then 0.5 ml of methanol and 4 ml of toluene scintillator - Triton

X-100 (2 : 1) mixture were added and counted as before [11]. Each experiment was done in triplicate, and the results are expressed as percentage of the controls, i.e. the untreated samples precipitated with TCA as above.

Efficiency of TCA precipitation of [^{14}C] NAC irreversibly bound to nucleic acids was estimated following precipitation of 0.2 ml aliquots of the purified complexes with 10% TCA at 0°C for 3 h, washing with 5% TCA and ethanol, solubilization with Hyamine and counting as described above. The controls were the samples directly spotted and dried onto GF/C filters prewashed with 5% TCA and ethanol. The filters were placed in counting vials and subjected to Hyamine treatment and counted (see above). The efficiency of TCA precipitation of DNA was estimated by phosphorus determination [3] of the yield of DNA retained on GF/C filters following TCA precipitation.

Stability of [^{14}C] NAC-DNA in the presence of SDS was assayed by incubation of 170-210 μg of the complex in 3 ml with SDS (2.5%) and NaCl (0.25 M) at 37°C for 3 h followed by filtration through Sephadex G-100 column (1.5 x 26 cm) equilibrated with 0.05 M phosphate buffer, pH 7.4. Absorbance at 260 nm and the radioactivity in the eluate was measured. The amount of radioactivity in the main peak corresponding to the high-molecular weight material was expressed as a percentage of the total radioactivity recovered from the column.

Assay of stability of complexes of NAC with proteins. The efficiency of TCA precipitation of NAC-albumin complex was followed by protein determination [7] and radioactivity measurements under the conditions described for the nucleic acids complexes.

RESULTS AND DISCUSSION

Thiol-dependent binding of NAC to nucleic acids. Incubation of NAC with DNA in the presence of sulfhydryl compounds leads to tight irreversible binding of the drug. General properties of the reaction may be summarized as follows. The yield of the adduct(s) formed in the presence of DTT is considerably higher than that with the other thiols tested: merceptoethanol [5, 9] or cysteine (unpublished observation). As shown on Fig. 1 the binding of NAC depends on DTT concentration, the optimum being observed at 2-5 mM. Dependences of the reaction on time and on ionic strength

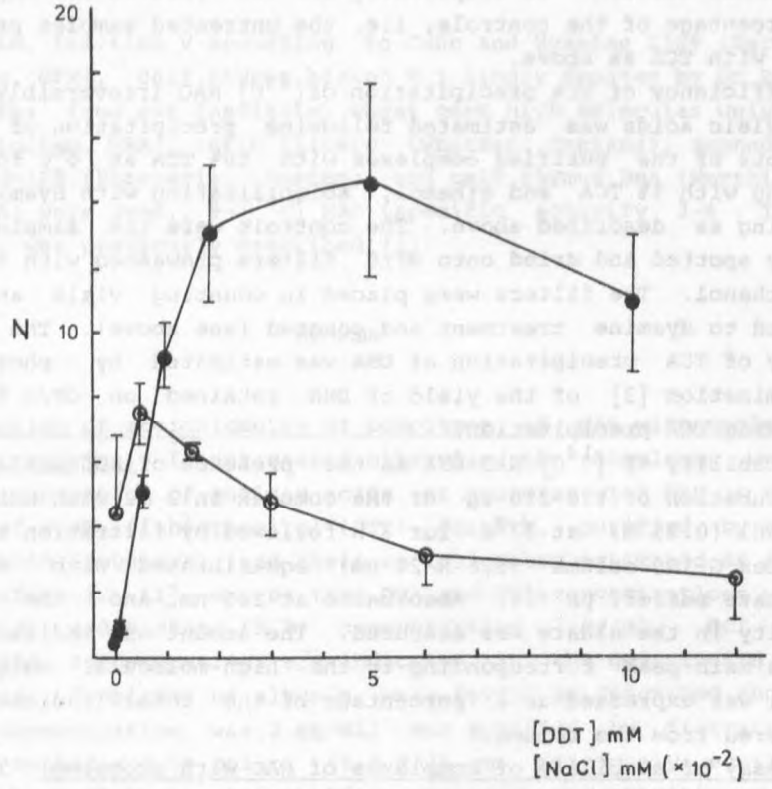

Fig. 1. Effect of salt and DTT concentration on NAC binding to DNA
Complexes were formed by incubation of DNA (400 µg/ml) with [^{14}C] NAC (0.1 mM in the presence of increasing concentration of NaCl (⊙) or DTT (●)

Rys. 1. Wpływ stężenia soli i DTT (ditiotreitolu) na wiązanie NAC z DNA
Kompleksy uzyskano podczas inkubacji DNA (400 µg/ml) z [^{14}C] NAC (0,1 mM) w obecności wzrastającego stężenia NaCl (⊙) lub DTT (●)

are shown on Figs. 1-2. The adduct formation with native and denatured calf thymus DNA and RNA does not differ more than by a factor of two [2].

Stability of NAC complexes with DNA and RNA. The following facts indicate that substantial amount of the drug in the purified complex is actually covalently bound to nucleic acids (Tab. 1): i) over 80% of the radioactivity is insoluble in TCA and this amount is close to the efficiency of DNA (90,7%) and RNA (87.3%)

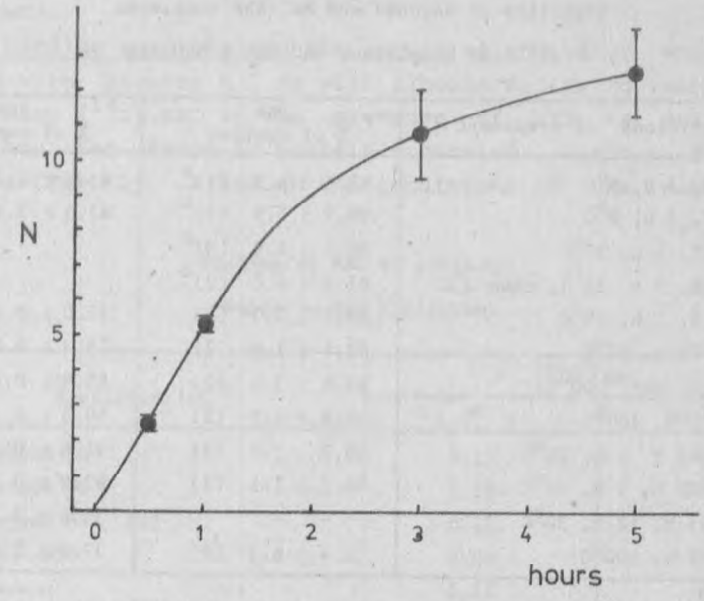

Fig. 2. Time course of NAC binding to DNA
Native DNA at DNA concentration of 400 µg/ml with [^{14}C] NAC (0.097 mM) and DTT (2 mM) was incubated for the time indicated

Rys. 2. Wpływ czasu na wiązanie NAC z DNA
Natywne DNA w stężeniu 400 µg/ml było inkubowane z [^{14}C] NAC (0,097 mM) i DTT (2 mM) przez wskazany czas

precipitation found under these conditions; ii) neither 7 M urea nor thermal or akaline denaturation considerably affect the efficiency of precipitation of [^{14}C] NAC-DNA with TCA; iii) 95.5% of [^{14}C] NAC from the complex with DNA migrates with high molecular material on Sephadex column after the incubation with SDS and over 80% of both the labelled drug and DNA from the complex co-precipitates with SDS in the presence of 1 M NaCl (Tab. 1); iv) as shown already [12] the NAC-DNA complex is resistant to high salt/phenol treatment. Decomposition of the high molecular material is observed following heating at pH 7.5 or pH 1.7 for 1 h. Other properties of the complexes are also summarized in Tab. 1.

The product of the reaction of NAC with RNA is somewhat less stable during the incubation at pH 7.5 at 37°C or upon heating at 100°C (e.g. only 66% is precipitated with TCA following he-

Table 1

Stability of NAC-DNA and NAC-RNA complexes

Stabilność kompleksów NAC-DNA i NAC-RNA

Conditions of treatment	DNA % of control	RNA % of control
TCA, 10%, 3 h, 0°C	87.4 ± 4.9 (7)[a]	83.5 ± 2.7 (2)[a]
TCA, 10%, 3 h, 0°C	90.7 ± 5.5 (4)[b]	87.3 ± 5.1 (3)[b]
SDS, 2.5%, 3 h, 37°C	95.5 ± 4.9 (3)[c]	-
SDS, 2.5%, 3 h, 37°C, then 0°C	83.5 ± 4.0 (2)[d]	-
urea, 7 M, 1 h, 20°C	85.2 ± 3.3 (5)	76.0 ± 6.1 (4)
pH 7.5, 70 h, 37°C	81.4 ± 1.6 (2)	75.3 ± 0.9 (2)
pH 7.5, 10 min, 100°C	89.0 ± 3.0 (2)	65.9 ± 0.6 (2)
pH 7.5, 1 h, 100°C	57.8 ± 9.7 (2)	50.3 ± 1.5 (2)
NaOH, 0.01 M, 1 h, 20°C	98.2 ± 2.9 (3)	96.6 ± 0.7 (2)
NaOH, 0.05 M, 1 h, 20°C	95.7 ± 1.1 (3)	93.9 ± 0.3 (2)
NaOH, 0.5 M, 18 h, 30°C	-	32.6 ± 1.3 (4)
pH 1.7, 1 h, 100°C	22.4 ± 6.1 (3)	37.2 ± 5.8 (5)

[a, b] Efficiency of precipitation of NAC-DNA and NAC-RNA complexes with TCA estimated by radioactivity measurements (a) and phosphorus determination (b).

[c] NAC-DNA complex following treatment with SDS was analyzed on Sephadex G--100 column.

[d] NAC-DNA treated as (c) but in the presence of 1 M NaCl instead of 0.25 M NaCl then cooled to 0°C. The material co-precipitated with SDS contained over 80% of DNA and indicated amount of radioactivity.

N o t e: In all the experiments except these denoted with superscripts a-d the samples of NAC-DNA (or NAC-RNA) were treated as indicated then precipitated with TCA and counted. The results are expressed as percentage of the corresponding controls, i.e. the untreated NAC-DNA or NAC-RNA complex. Averages of two or more experiments are given (as indicated in the parantheses) ± range or standard deviations values.

ating in a boiling water bath for 10 min.; see Tab. 1). Rather surprisingly [^{14}C] NAC-RNA is resistant in the highly alkaline medium. As much as 33% of the radioactivity remains acid insoluble under the conditions which are used to hydrolyze RNA (incubation with 0.5 M NaOH for 18 h). Similar amount of the drug is observed at the origin of the paper strip when the alkaline hydrolysate of NAC-RNA is subjected to chromatography in ethanol/ammonium acetate (7 : 3) while the rest of radioactivity migrates with nucleotides in two peaks. The yield of phosphorus RNA precipitated with TCA following incubation with 0.5 M NaCl is however similar

within the accuracy of the determination for NAC-RNA and free RNA (not shown).

NAC binding to proteins. The amount of the drug which is co-purified with histone H 1 or with albumin following incubation of protein with [^{14}C] NAC in the presence of DTT is considerably higher than that found in protein incubated without sulfhydryl compounds (Tab. 2). Although the conditions of the reaction with

T a b l e 2

Binding of NAC to proteins

Wiązanie NAC z białkami

Specification		[^{14}C] NAC	
		mM	nmole/mg
I	Histone H1	0.13	5.1
II	Histone H1	0.20	9.2
	Histone H1 - DTT	0.20	1.4[a]
	DNA - DTT	0.20	0.4[b]
III	Albumin	0.14	6.6
IV	Albumin	0.14	9.0
	Albumin - DTT[a]	0.14	0.4

[a] The control without DTT.

[b] DNA incubated with [^{14}C] NAC but without DTT. The amount of [^{14}C] NAC bound to DNA in the presence of DTT is about 50 nmole/mg under these conditions.

N o t e: Concentration of [^{14}C] NAC (mM) during the complex formation and the amount of the drug bound to protein (nmole/mg) is indicated.

proteins are somewhat different than those used for DNA the approximative level of binding of NAC to proteins per mass unit is about tenfold lower than that found for DNA. It is similar for the two proteins as different as histone and albumin (Tab. 2). Although the protein - drug complexes were less thoroughly studied than those with nucleic acids following facts indicate that covalent binding does occur: i) there is no release of the label from the purified [^{14}C] NAC albumin complex when it is incubated with an excess of the unlabelled drug - 97-99% of the radioactivity is eluted with the protein peak from Sephadex G-50 column; ii) over 90% of the radioactivity is co-eluted with protein peak following incubation of the complex with SDS.

ACKNOWLEDGEMENTS

The authors thank Dr. W. A. Denny for reading a draft of this manuscript and helpful comments and to Miss M. Affeltowicz for technical assistance. This work is supported by grant No 2304 of the Polish National Cancer Program.

REFERENCES

[1] Denny W. A., Baguley B. C., Cain B. F., Waring M. J. (1983), Molecular aspects of anticancer drug action eds S. Neidle, M. J. Waring, Macmillan, London, 1-34.

[2] Gniazdowski M., Ciesielska E., Szmigiero L. (1981), Chem.-Biol. Interactions, 34, 355-366.

[3] Gniazdowski N., Filipowicz B., (1969), Anal. Biochem., 32, 204-209.

[4] Gniazdowski M., Filipski J., Chorąży M. (1979), Antibiotics, ed. F. E. Hahn New York, Vol. V, Part 2, 275-297.

[5] Gniazdowski M., Szmigiero L., Śląska K., Jaros-Kamińska B., Ciesielska E. (1975), Mol. Pharmacol., 11, 310-318.

[6] Gniazdowski M., Szmigiero L., Wilmańska D. (1982), Cancer Lett., 15, 73-79.

[7] Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J. (1951), J. Biol. Chem., 193, 265-275.

[8] Pawlak K., Matuszkiewicz A., Pawlak J. W., Konopa J. (1983), Chem.-Biol. Interactions, 43, 131-149.

[9] Szmigiero L., Gniazdowski M. (1981), Arzneim. Forsch.-Drug Res., 11, 1875-1877.

[10] Szmigiero L., Śląska K., Ciesielska E., Jaros-Kamińska B., Gniazdowski M., (1977) Acta Biochim. Polon., 24, 35-44.

[11] Śląska K., Szmigiero L., Jaros-Kamińska B., Ciesielska E., Gniazdowski M. (1979), Mol. Pharmacol., 16, 287-296.

[12] Wilmańska D., Małagocka E., Szmigiero L., Gniazdowski M. (1984), Biochim. Biophys. Acta, 782, 285-294.

Department of General Chemistry
Institute of Physiology and Biochemistry
Medical Academy of Łódź

Małgorzata Czyż, Kazimierz Studzian, Andrzej Wasilewski,
Aleksandra Poziomska, Dorota Wilmańska, Marek Gniazdowski

WIĄZANIE KOWALENCYJNE NITRAKRYNY (LEDAKRIN, C-283) Z KWASAMI NUKLEINOWYMI I BIAŁKAMI

W obecności związków sulfhydrylowych lek przeciwnowotworowy nitrakryna (Ledakrin, C-283) tworzy nieodwracalne kompleksy z DNA i RNA. Przedstawiono charakterystykę stabilności kompleksów w różnych warunkach. Szereg faktów wskazuje na kowalencyjny charakter wiązania pomiędzy Ledakrinem a kwasami nukleinowymi: 1) [^{14}C] NAC znajdujący się w oczyszczonym kompleksie jest wytrącany kwasem trójchlorooctowym z podobną wydajnością jak DNA czy RNA; 2) kompleksy nie dysocjują w roztworach 7 M mocznika i siarczanu dodecylu sodu (SDS) oraz w wysokiej sile jonowej; 3) zarówno termiczna, jak i alkaliczna denaturacja nie wpływa znacząco na wydajność strącania kompleksu [^{14}C] NAC-DNA przy pomocy kwasu trójchlorooctowego. Ponadto stwierdzono, że w obecności ditiotreitolu (DTT) [^{14}C] NAC wiąże się w sposób nieodwracalny z albuminą i histonem, chociaż ilość związanego leku jest dziesięciokrotnie niższa niż w przypadku DNA.