Biochemical Pharmacology, Vol. 36, No. 23, pp. 4169–4171, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

^{32}P-Post-labelling analysis of DNA adducts formed by antitumor 1-nitro-9-aminoacridines with DNA of HeLa S_3 cells*

(*Received* 20 *March* 1987; *accepted* 9 *June* 1987)

A number of derivatives of 1-nitro-9-aminoacridine, synthesized and studied in this department, show potent cytotoxic and antitumor properties [1, 2]. One of these compounds, 1-nitro-9(3′-dimethylaminopropylamino)-acridine, has been used clinically in Poland under the name of Ledakrin (synonyms: Nitracrin, C-283) [3, 4]. Another one, C-857, is in the process of being accepted for clinical studies.

Previous investigations on the mode of action of 1-nitro-9-aminoacridines carried out in our laboratory demonstrated that Ledakrin is a latent form of the drug which upon metabolic activation binds covalently to DNA and other cellular macromolecules [5, 6]. Moreover, Ledakrin as well as other 1-nitro-9-aminoacridines was found to be a potent DNA cross-linking agent as revealed by means of denaturation–renaturation methods [7]. The ability of these compounds to produce covalent interstrand cross-links has been postulated to represent the crucial event responsible for their antitumor properties [7].

In this paper we present further data on covalent binding to DNA and DNA adduct formation by Ledakrin and other derivatives of nitroacridine. Such studies were previously restricted to Ledakrin because only this compound was available in an isotopically labelled form. Since the ^{32}P-post-labelling test circumvents this requirement we were able to extend the scope of our investigations to other nitroacridines.

Materials and methods

Chemicals. Ledakrin and its analogues were kindly provided by the late Professor Andrzej Ledóchowski from this Department. Hydroxylapatite (Bio-Gel HTP, DNA grade) was from Bio-Rad. Micrococcal endonuclease (Sigma) was dissolved in solution of spleen exonuclease (Boehringer) dialyzed against 40 mM sodium succinate, 20 mM $CaCl_2$, pH 6. T4 polinucleotide kinase (Pharmacia), potato apyrase (Sigma) and proteinase K (Merck) were used without purification. Deoxyribonucleotides were from Pharmacia, (PEI)-cellulose †thin layers were from Merck, γ-[^{32}P]-ATP (3000 Ci/mmol) was from Amersham International.

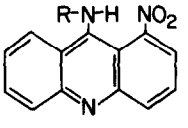
DNA isolation and adduct analysis. HeLa S_3 cells were grown in spinner culture as described previously [8]. Cell suspensions were incubated with the nitroacridines as specified in the legends to the figures. DNA isolation was performed by the modified hydroxylapatite procedure [9]. Briefly, HeLa S_3 cells were collected by centrifugation and homogenized in Dounce homogenizer in 1% SDS followed by incubation with proteinase K (250 μg/ml, 38°, 90 min). DNA was purified using hydroxylapatite (1 g/50 mln cells) and then extensively dialyzed against distilled water (3 × 30 min, 4 l/sample) by thin layer dialysis [10]. DNA solutions were stored at 0° until submitted to a further procedure (usually 2–3 days). DNA adducts were analyzed by ^{32}P-post-labelling technique [11, 12]. The DNA was hydrolyzed to nucleotides, the nucleotides were converted to ^{32}P-labelled deoxyribonucleotide-3′,5′-diphosphates. The reaction mixture consisted of 2 μl of freshly prepared DNA digest, 4 μl of ^{32}P-ATP (150 μCi), 3 μl of 0.1 M Bicine-NaOH, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermine, pH 9, and 11.5 U of T4 polinucleotide kinase. The reaction mixture was incubated for 30 min at 38°, then 2 μl of 150 mM ATP was added and the incubation was performed for an additional 60 min. The individual adducts were resolved by 4-directional TLC [12]. Autoradiography was performed at −20° for 2–3 days after which the adduct spots and adjacent areas were excised and their radioactivity was determined in toluene-based scintillation fluid. The concentration of the adducts was determined from the relative adduct labelling index as defined by Gupta *et al.* [11].

Results and discussion

It was demonstrated in our laboratory that derivatives of 1-nitro-9-aminoacridine are potent DNA cross-linking agents, however, the requirement of radiolabelled derivatives limited DNA-binding studies to Ledakrin. The application of ^{32}P-post-labelling test allowed us to avoid this restriction and to perform such studies also for other derivatives of nitroacridine.

In this paper we present the results of the experiments on DNA adduct formation by Ledakrin and two other compounds, denoted C-857 and C-1006, which differ from Ledakrin only in the structure of the side chain. The chemical formulae and EC_{50} values against HeLa S_3 cells are shown in Fig. 1.

Figure 2b presents an autoradiogram from the Ledakrin-modified DNA of HeLa S_3 cells. The conditions of HeLa S_3 treatment (2 μg/ml, 4 hr) match the ones for which in previous experiments, performed by a different method requiring the drug in a radiolabelled form [6], the maximal extent of covalent binding was detected. In the chromatogram, at least five spots were detected that were not observed in control DNA (Fig. 2a). The spot number 5 constituted about 37% of the total adduct radioactivity. The level of modification of DNA was 1 adduct in 440 nucleotides, as calculated from the total adduct radioactivity of the normal nucleotides. This result is in close agreement with the previous data, obtained by different



COMPOUND	R	EC_{50}(nM)
LEDAKRIN	$-(CH_2)_3N(CH_3)_2$	3.7
C-857	$-(CH_2)_2OH$	0.5
C-1006	$-(CH_2)_3NH(CH_2)_5CH_3$	13.0

Fig. 1. Structures of the 1-nitro-9-aminoacridines studied and their cytotoxic activities (EC_{50}) against HeLa S_3 cells. EC_{50} values are drug concentrations inhibiting by 50% cell growth after 72 hr treatment as described elsewhere [7].

* Part of this work was presented at the 14th International Cancer Congress, 21–27 August, 1986, Budapest, Hungary, Proceedings, Abstract No 2043.

† Abbreviations used: EC_{50}, drug concentration inhibiting by 50% cellular growth (cytotoxic activity); (PEI)-cellulose, polyethylene-imine-cellulose; TLC, thin layer chromatography; BICINE, *N,N*-bis(2-hydroxyethyl)-glycine.

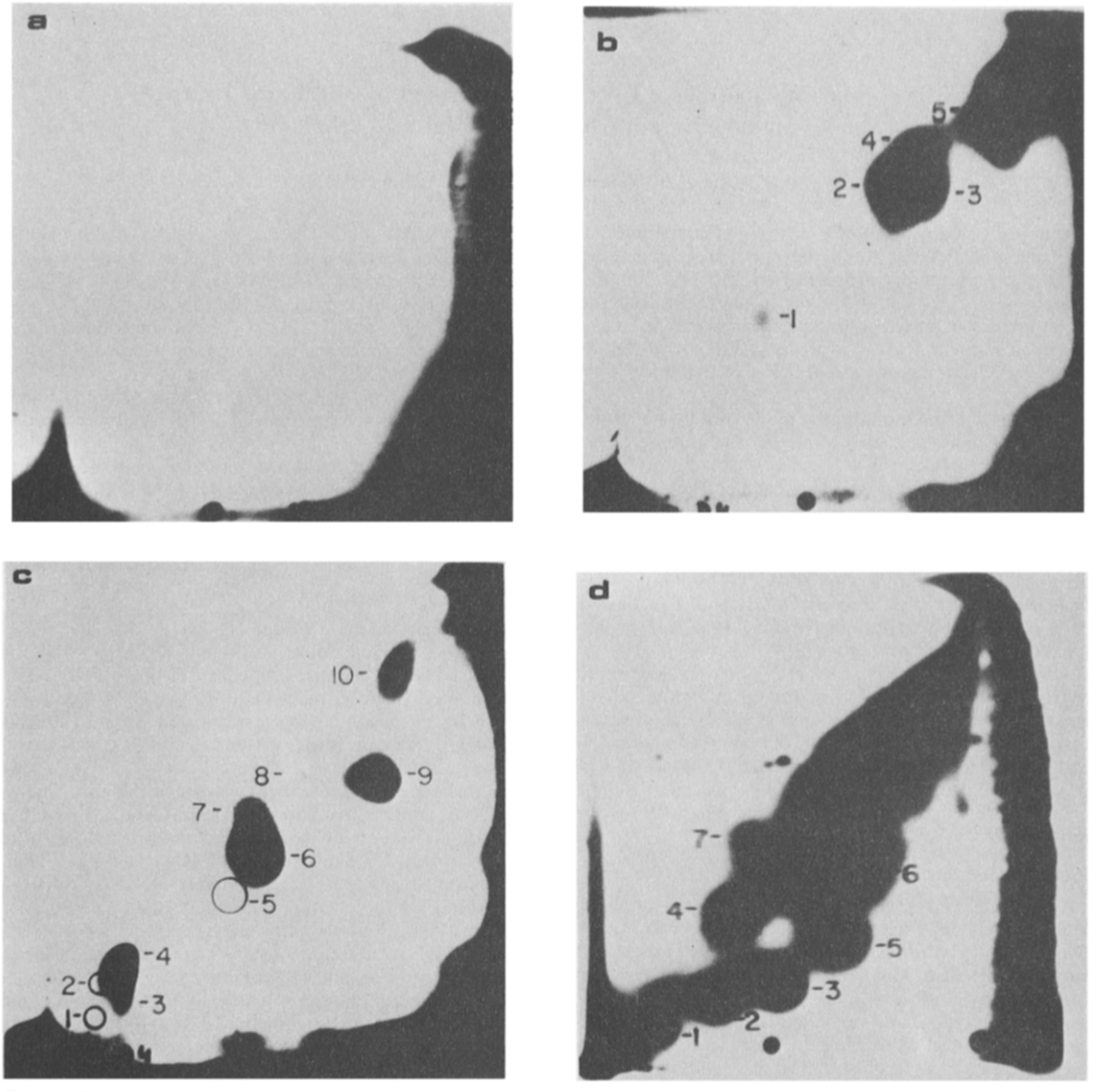


Fig. 2. Autoradiograms of DNA from HeLa S_3 cells treated with the derivatives of 1-nitro-9-aminoacridine, (a) control non-treated cells, (b) 2 μg/ml Ledakrin, (c) 0.22 μg/ml C-857 and (d) 7.4 μg/ml C-1006. The cells were incubated with the drugs at concentrations constituting 1360 × EC_{50} for 4 hr. The DNAs were isolated, digested to dNps, which were subjected to ^{32}P-labelling and the individual adducts were resolved by TLC as described in Materials and Methods. Approximately 120 μCi was applied to the chromatograms. Spots were visualized by autoradiography at −20° for 3 days.

procedure using [^{14}C]-Ledakrin, where the maximal extent of covalent binding to HeLa S_3 cells DNA was demonstrated as being 1 molecule of the drug per 370 DNA base pairs and five drug-nucleoside adducts were separated during fractionation of the enzymatic digest of DNA isolated from Ehrlich ascites cells exposed *in vivo* to Ledakrin as revealed by field desorption mass spectrometry [6].

The biologically inactive 2-nitro isomer of Ledakrin, denoted C-264, even at a concentration of up to 50 times higher than the one applied in the case of Ledakrin, did not give rise to any adducts. An autoradiogram of DNA from HeLa S_3 cells exposed to C-264 is the same as that of DNA from control cells. This result shows clearly that a nitro group at position 1 of an acridine ring is absolutely necessary for covalent binding of nitroacridines to DNA. Hence, it means that this capability of covalent binding to DNA is a prerequisite for biological activity of 1-nitro derivatives since the 2-nitro isomer of Ledakrin is biologically inactive.

Moreover, as the 2-nitro isomer of Ledakrin much better intercalates into DNA than Ledakrin itself [13], such a finding indicates that intercalation is of negligible, if any, biological significance.

^{32}P-fingerprints of DNA adducts from HeLa S_3 cells treated with 1-nitro derivatives, C-857 and C-1006, are shown in Fig. 2c and Fig. 2d, respectively. The conditions applied were the same, in terms of multiplicity of EC_{50} values and exposure time as in the case of Ledakrin. An autoradiogram from C-857 treated HeLa S_3 cells shows 10 extra spots; two of them numbered 4 and 6 accounted for about 50% of the total adduct radioactivity. Under the applied conditions one adduct was detected per 330 nucleotides.

The treatment of HeLa S_3 cells with C-1006 resulted in

a formation of several different adducts (about 20) whose number was hard to establish due to small differences in their R_f values. The extent of modification amounted to one adduct per about 1000 nucleotides.

In conclusion the study indicates that 1-nitro-9-aminoacridines are able to covalently bind to DNA of HeLa S_3 cells to an exceptionally high degree, from 1 molecule of the drug per 330 to 1000 nucleotides for C-857 and C-1006, respectively. As is shown in Fig. 1, the compounds studied differ in their EC_{50} values, thus, in their cytotoxic activity. The ability to covalently bind to DNA seems to be correlated with cytotoxic activities since when the HeLa S_3 cells were treated with the drugs at the concentrations constituting the same multiplicity of EC_{50} ($1360 \times EC_{50}$ in our experiments), the degree of covalent binding to DNA remained approximately the same for all the compounds. This means that more biologically active derivatives of 1-nitro-9-aminoacridine are also more potent DNA binding agents.

The position of a nitro group is very important since, as it was shown, 1-nitro derivatives are able to bind covalently to DNA while the 2-nitro isomer of Ledakrin did not give rise to any DNA adducts. As both 1- and 2-nitroacridines intercalate into DNA and the biologically inactive 2-nitro derivative does not form DNA adducts, it means that not intercalation but covalent binding to DNA is a prerequisite for biological activity of 1-nitro-9-aminoacridines. On the other hand, since all the 1-nitro derivatives studied possess the same nitro-substituted acridine core, it must be the structure of the side chain that is responsible for the degree of covalent binding to DNA. The side chain also has influence on the number of different DNA adducts that a given derivative can form (e.g. 5 DNA adducts for Ledakrin and at least 20 for C-1006). The autoradiograms presented in this paper do not allow one to ascertain whether any of the DNA adducts detected are common for all investigated compounds. It is interesting, however, that the number of DNA adducts does not correspond to cytotoxic activity.

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Acknowledgements—The authors wish to thank A. Składanowski M.Sc. and M. Bontemps-Gracz M.Sc. for help with the preparation of the manuscript. These studies were supported by National Cancer Programme Grant CPBR 11.5/115.

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Biochemical Pharmacology, Vol. 36, No. 23, pp. 4171–4173, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Studies on *N*-demethylation of methamphetamine by means of purified guinea-pig liver flavin-containing monooxygenase

(*Received* 17 *March* 1987; *accepted* 23 *June* 1987

The metabolism of methamphetamine (MP) has been studied extensively in mammals including man [1]. *N*-Demethylation, one of the major metabolic pathways of MP, has been recognized to proceed via at least two independent routes. One is the *C*-hydroxylation pathway catalyzed mainly by cytochrome (cyt.) P-450 [2] and another is *N*-hydroxylation pathway catalyzed mainly by flavin-containing monooxygenase (FMO) [3]. Our previous work [4] revealed that *N*-demethylation of MP proceeds mainly by the *N*-hydroxylation pathway in guinea pigs. In the present study, the role of FMO in *N*-hydroxylation of MP and *N*-demethylation of *N*-hydroxy-MP was investigated using liver microsomes and purified FMO from guinea-pig liver microsomes.

Materials and methods

Chemicals. d-MP hydrochloride was purchased from Dainippon Pharmaceutical Co., Osaka. Neutral oxalates of *N*-hydroxy-MP and *N*-hydroxyamphetamine (*N*-hydroxy-AP) were synthesized by the method of Coutts *et al.* [5]. All other reagents used were from the sources described elsewhere [4, 6] or of the highest quality commercially available.

Purification of FMO. Liver microsomes of Hartley guinea pigs (250–350 g) prepared by the method described earlier [4] were solubilized with 1.0% Emulgen 911 in Buffer A (10 mM potassium phosphate (pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1.0 mM EDTA and 20% glycerol). Solubilized supernatants obtained by ultracentrifugation at 105,000 *g* for 60 min were diluted with 4 vol. of Buffer A, and applied onto Procion-Blue Sepharose 4B (5 cm i.d. × 25 cm) [7] equilibrated with 0.2% Emulgen 911 in A buffer. FMO was eluted with a linear gradient of KCl from 0 to 1.0 M in the equilibration buffer. Fractions containing FMO (0.2–0.3 M KCl) were pooled and dialyzed against equilibration buffer. These were then applied onto DEAE-Sephacel column (Pharmacia Fine Chemical Co., 2 cm i.d. × 10 cm) equilibrated with 0.2% Emulgen 911 in Buffer A, and eluted with a linear gradient of KCl from 0 to 1.0 M in the equilibration