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Short communication

Inhibition of mammalian topoisomerase I by 1-nitro-9-aminoacridines. Dependence on thiol activation*

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The cytotoxic activity, susceptibility to thiol activation and ability of eight 1-nitroacridine derivatives to stabilize the topoisomerase I-DNA cleavable complex, were compared. Among the acridines tested three compounds exhibited high ability to stabilize the cleavable complex. This ability was correlated with susceptibility to thiol activation as well as with cytotoxic activity. Our results suggest that 1-nitroacridine-DNA adducts interfering with topoisomerase I action may contribute to the lethal effects of some 1-nitroacridine derivatives.

1-Nitroacridine derivatives are of interest as potential bioreductive anticancer agents. The most prominent member of this group, nitracrine (1-nitro-9-[3,3-*N,N*-dimethylaminopropylamino]acridine, Fig.1) exhibits high cytotoxic and antitumour activity in many experimental systems, selective cytotoxicity against hypoxic cells and can be used as a radiosensitizer. These properties may have interesting pharmacological implications as the presence of hypoxic cells in solid tumours can decrease the therapeutic efficacy of chemotherapy and radiotherapy for some malignant diseases [1]. We reported earlier that an anticancer drug nitracrine inhibited relaxation of pBR322 DNA catalysed by mammalian topoisomerase I (topo I) [2]. This finding seems to be of interest as topo I enzymes are ubiquitous and play a pivotal role in DNA transcription, replication and repair. Furthermore, eucaryotic topo I has been identified as a potential target for anticancer therapy. There is much evidence that anticancer camptothecin derivatives used clinically stabilize a critical intermediate step of topo I catalysis, at which the enzyme induces a single-strand break in the substrate DNA and is covalently linked to it. This step is called a cleavable complex as it can be hydrolyzed by proteinase K digestion yielding a

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Abbreviation: topo I, topoisomerase I

nicked DNA substrate [3]. We found that nitracrine stabilizes the topo I cleavable complex in a manner similar to that of camptothecin action [4]. This stabilization occurs upon reductive activation of the drug by dithiothreitol, leading to covalent binding of a nitracrine intermediate to DNA.

In this report we examine a series of nitracrine analogues with varying side chains and two bis-1-nitroacridines to evaluate the relationships between susceptibility of these compounds to reductive thiol activation and their ability to stabilize the cleavable complex of topo I, as well as their cytotoxic activity.

MATERIALS AND METHODS

Drugs and cytotoxicity studies. Structures of acridines are shown in Fig. 1. Bis-1-nitroacridines and 4-methylnitracrine were synthesized at the Cancer Research Laboratory, University of Auckland and kindly donated by Dr. W.A. Denny. Other compounds were a gift from Professor J. Konopa from the Technical University of Gdańsk. Mouse leukemia L1210 cells were cultured in RPMI 1630 medium (Sigma-Aldrich, U.S.A.) supplemented with 10% of foetal calf serum (Gibco, U.S.A.), gentamycin (50 μg/mL) and

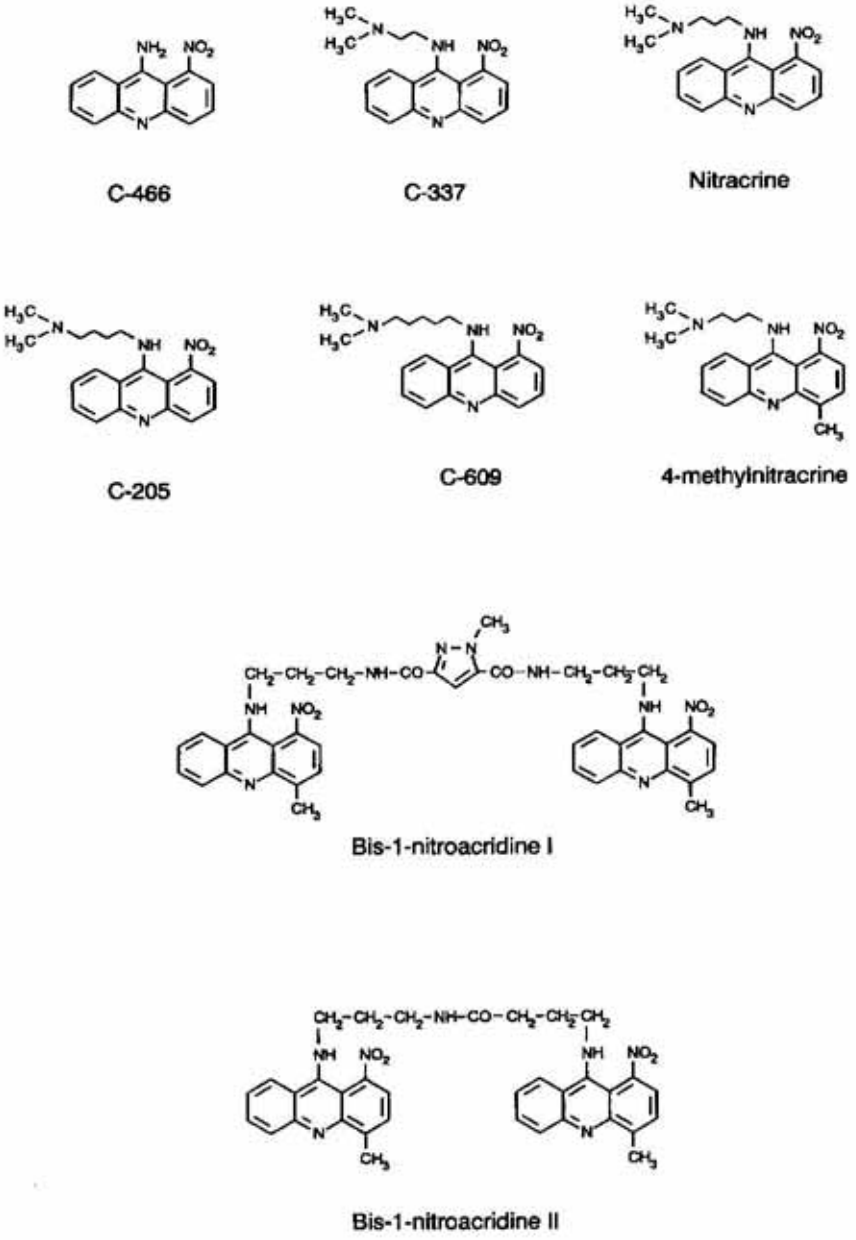

Figure 1. Structures of the acridines.

0.02 M Hepes buffer (Gibco, U.S.A.). Cytotoxic effects were assayed by measuring the inhibitory effects on L1210 cell proliferation. In this assay cells were seeded in 3 mL aliquots in 6 mL tissue culture tubes (Corning, U.S.A.) at a concentration of 2×10^4 cells/mL and exposed to drugs for 1 h at 37°C. After 72 h of incubation in a fresh growth medium the cell number relative to control was determined by the tetrazolium dye method [5].

The susceptibility of 1-nitroacridines to thiol activation was measured by a spectrophotometric method. As reduction of the nitro group of nitroacridines leads to formation of hydroxylamine derivatives with absorption maximum at 570 nm, the increase of light absorption at this wavelength occurring after 1 h incubation of 0.1 mM drugs with 2 mM dithiothreitol (DTT) at 37°C was taken as the reduction indicator.

The "cleavage assay" was carried out in a system containing partially purified topoisomerase I from L1210 cells and supercoiled pBR322 DNA in the absence or presence of 2 mM dithiothreitol, under conditions described by Hertzberg *et al.* [6]. Reaction volumes were typically 20 μL. After 30 min of incubation at 37°C, the reaction was stopped by adding sodium dodecylsulfate and proteinase K. After an other 60 min of incubation at 37°C, 5 μL of gel loading buffer was added. Samples were loaded on 0.9% agarose gel and electrophoresis was run as described by Ciesielska & Szmigiero [7]. Gels were photographed by a CCD camera under UV light. The densitometric analysis was performed by an image analysis software (Pharmacia-LKB).

RESULTS AND DISCUSSION

Metabolic reduction of 1-nitroacridines leads to formation of an active intermediate that can bind covalently to macromolecules [1, 8]. This intermediate of unknown structure is very unstable whereas the major product of nitracrine metabolism, a 1-hydroxylamine derivative, is relatively stable. As 1-hydroxylaminoacridines are of violet colour, changes in their concentration can be easily assayed by a colorimetric measurement and used for monitoring of the reduction process. Among the tested acridines three compounds exhibited high susceptibility to thiol activation. As shown in Fig. 2, nitracrine and its analogues C-205 and C-609 reacted with dithiothreitol giving products with absorption maximum at 570 nm. Neither 4-methylnitracrine (Fig. 2) or the analogues of nitracrine with 9-alkylamino side chain containing less than 3 methylene groups (C-466 and C-337), nor bis-1-nitroacridines exhibited any increase of light absorption at 570 nm (Table 1). Differences in the reactivity of nitroacridines with the reducing agent dithiothreitol may be dependent on the tautomeric form of a nitroacridine and its reducing potential. 1-Nitroacridines can exist as either aminoacridine or iminoacridan tautomers. The equilibrium between these two forms depends on the structure and electronic properties of acridine substituents at position 9, and influences the biological activity of this class of compounds. These acridines which tend to exist at pH 7 as the iminoacridan tautomer exhibit higher hypoxia-selective cytotoxicity [9]. Susceptibility of 1-nitroacridines to thiol activation seems to be also linked with the presence of the iminoacridan tautomer. Nitracrine, C-205 and C-609 which can adopt the iminoacridan form [10] reacted with dithiothreitol whereas C-466 which exists as an aminoacridine was resistant to thiol activation (Fig. 2 and Table 1). C-337 also exhibited resistance to thiol activation. This result is rather unexpected as for the latter acridine the iminoacridan tautomer is the favoured form. A possible explanation of poor reactivity of C-337 with dithiothreitol is a much lower reduction potential of C-337 when compared with that of analogues possessing a longer side chain [9]. Other compounds resistant to thiol activation were substituted by methyl group at position 4. This modification is known to lower the reduction potential of 1-nitroacridines [10].

Nitracrine inhibited relaxation of supercoiled pBR322 DNA by topoisomerase I and it also increased the amount of nicked DNA. This effect was observed only upon reductive thiol activation of this acridine. As shown in

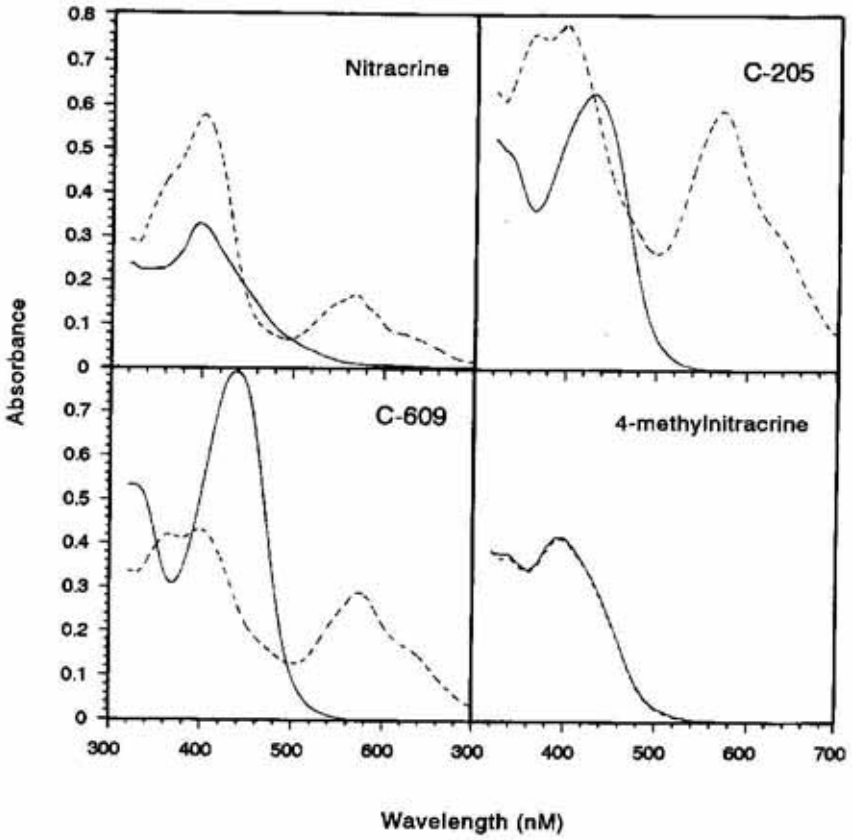

Figure 2. Absorption spectra of 0.1 mM nitroacridines dissolved in 0.05 mM Tris/HCl, pH 7.

Solid lines, acridines not incubated with dithiothreitol; dashed lines, acridines incubated for 1 h at 37°C with 2 mM dithiothreitol.

lane 2 of Fig. 3A, topoisomerase I in the absence of acridines converted supercoiled DNA (CC) into relaxed form (R). In the absence of thiols nitracrine was inactive and neither inhibited the relaxation of pBR322 DNA nor enhanced the amount of its nicked form (Fig. 3A, lanes 3–7). When substrate DNA was incubated with topoisomerase I and nitracrine in the presence of 2 mM dithiothreitol the increase in the amount of nicked DNA (OC) was observed (Fig. 3A, lanes 8–12). This result indicates that thiol activated nitracrine stabilizes the cleavable complex of topoisomerase I with DNA. At the

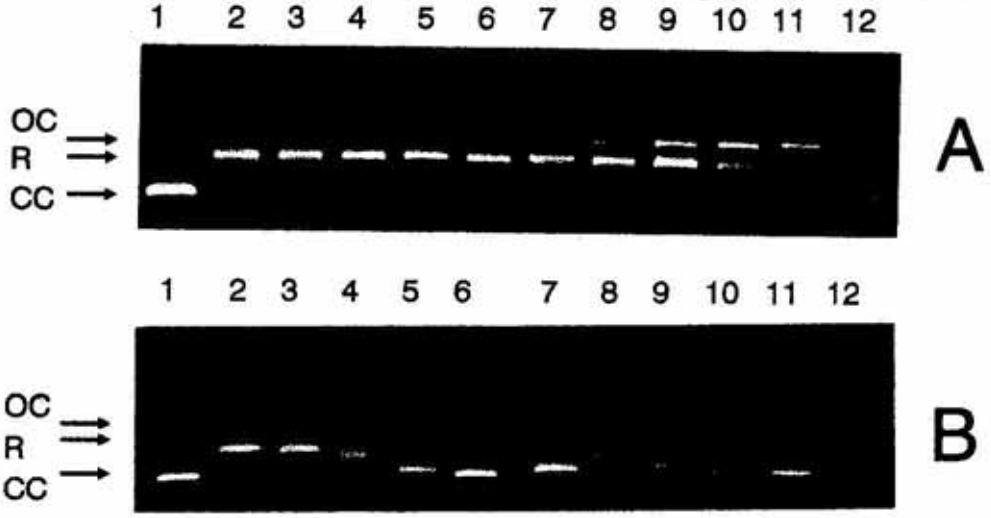

Figure 3. Effect of nitracrine (A) and C-466 (B) on relaxation of pBR322 DNA catalyzed by topoisomerase I from L1210 cells.

Lanes: 1, substrate DNA without topo I; 2, DNA + topo I (control); 3–7, DNA + topo I + acridine; 8–12, DNA + topo I + acridine + 2 mM dithiothreitol. DNA forms: OC, open circular (nicked); R, closed circular relaxed; CC, closed circular supercoiled. Acridines concentrations; lanes 3 and 8, 1 μM; lanes 4 and 9, 5 μM; lanes 5 and 10, 10 μM; lanes 6 and 11, 50 μM; lanes 7 and 12, 100 μM.

highest nitracrine concentrations the enhancement of the amount of nicked DNA was accompanied by the inhibition of DNA relaxation as bands of DNA corresponding to supercoiled DNA forms (CC) were found on the gel (Fig. 3A, lanes 11 and 12).

Other nitroacridines varied in their effects on the topoisomerase I catalyzed reaction. Compounds C-205 and C-609 exhibited an inhibitory effect on topo I similar to that of nitracrine (Table 1). The nitracrine analogue C-466 without an alkyl side chain in position 9 inhibited the relaxation of pBR322 DNA and ability to stabilize the topoisomerase I--DNA cleavable complex are compared in Table 1. Three compounds: nitracrine, C-205 and C-609 which undergo thiol activation and bind covalently to DNA possess a high cytotoxic potency and their ability to trap topoisomerase I at the cleavable complex step is higher than that of other nitroacridines. The fact that the thiol activated 1-nitroacridines stimulate of DNA cleavage catalyzed by topoisomerase I suggests that an intermediate of these compounds traps the aborted topoisomerase I-DNA complex.

Table 1. Cytotoxicity, susceptibility to thiol activation and ability of 1-nitroacridines to stabilize the cleavable complex of topoisomerase I with DNA

Compound	Cytotoxic activity (ED_{50}) (μM)	Susceptibility to thiol activation*	Ability to stabilize topo I-DNA cleavable complex**
C-466	21.6	0	1.5
C-337	22.0	0	4.6
Nitracrine	0.05	0.172	20.6
4-Methylnitracrine	0.86	0	2.2
C-205	0.02	0.593	15.6
C-609	0.03	0.295	14.5
Bis-1-nitroacridine I	0.81	0	3.4
Bis-1-nitroacridine II	1.13	0	2.5

*A_{540} of 0.1 mM acridine incubated with 2 mM dithiothreitol for 1 h at 37°C. **The increase of the amount (%) of nicked pBR322 DNA induced by 10 μM acridine in the presence of 2 mM dithiothreitol and topoisomerase I. ED_{50}, the acridine concentration (μM) effective in inhibiting by 50% the cell growth after 1 h exposure of L1210 cells to the drug.

but its effects was not dependent on thiol activation, and no increase in the amount of nicked DNA was noticed (Fig. 3B). The changes in the rate of migration of closed circular DNA observed in the gel seem to be related to the drug intercalation into DNA as C-466 compound possessing a flat aminoacridine structure can form a strong intercalative complex and unwind pBR322 DNA whereby disturbing the action of topoisomerase I. Such effects of acridine intercalating dyes have been described in the literature [11]. 4-Methylnitracrine, C-337 and bis-1-nitroacridines were poor topo I inhibitors both in the absence and presence of dithiothreitol (not shown).

The cytotoxic activity of the acridines tested, their susceptibility to thiol activation

Our results suggest that 1-nitroacridine-DNA adducts interfering with topoisomerase I action may contribute to the cytotoxic effects of some 1-nitroacridine derivatives. It is also possible that modification of a nitracrine molecule may lead to designing of derivatives directed more selectively to topoisomerase I.

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