Base specific interaction of reductively activated nitroimidazoles with DNA

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To exert biological activity, nitroimidazole drugs require reductive activation in vivo. Nucleic acids are susceptible to the activated drug in vitro and are presumably the major target in vivo. We carried out electrolytical reduction of several 5-nitroimidazoles at a controlled potential either in the presence or prior to the addition of DNA. Using a nucleotide sequence specific test to analyse cleavage products, specific interaction of the reduced nitroimidazole intermediate(s) towards the guanine residues is prominent. Since the strand scission depends on subsequent piperidine treatment, it can be concluded that the primary interaction between the activated drug and guanine is a covalent modification weakening the glycosidic bond.

DNA damage *Nitroimidazole* *Ornidazole* *Nucleic acid* *Reductive activation*

1. INTRODUCTION

Nitroimidazole drugs are extensively used against infections caused by anaerobic bacteria and protozoa [1,2]. They also received much attention in cancer therapy as radiosensitizers of hypoxic tumours and by their direct cytotoxic effects towards hypoxic cells [3]. The precise mechanism of action of these drugs is unknown, as well as their site(s) of interaction with cellular macromolecules. Most probably, nucleic acids are a major target [4].

Reduction of the nitro group is an essential step in the activation of nitroimidazoles. The resulting reactive intermediates are elusive, but in vitro generation of these intermediates in the presence of DNA has clearly shown a direct interaction of the activated nitromidazoles with DNA [5–8]. These experiments, however, did not reveal unambiguously the nature of the target site in the DNA. In an attempt to unravel this interaction, we used end-labelled DNA fragments as substrates for several activated nitroimidazoles. Reaction products were analysed by comparison with the appropriate chemical degradation sequencing ladders to assess the susceptibility of the DNA to the drug and its nucleotide and/or sequence specificity [9,10].

2. MATERIALS AND METHODS

2.1. *Enzymes, DNA and chemicals*

*Hin*fI and *Hae*III were purchased from New England Biolabs (Beverly, USA) and used as recommended. DNA polymerase I (Klenow) was from Boehringer (Mannheim). pGV001 is a deletion derivative of pBR327 [Volckaert et al., in preparation]. All chemicals were of analytical grade. Ornidazole [1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole], dimetridazole (1,2-dimethyl-5-nitroimidazole), ronidazole [1-methyl-2-[(carbamoyloxy)-methyl]-5-nitroimidazole] and MK-910 [2-(4-fluorophenyl)-1-methyl-5-nitroimidazole], carnidazole [*O*-methyl-{2-(2-methyl-5-nitroimidazol-1-yl)ethyl}-thiocarbamate], nimorazole [1-(2-*N*-morpholinylethyl)-5-nitroimidazole] were gifts from Roche

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2.2. *Preparation of the end-labelled DNA fragments*

DNA fragments were prepared by double digestion of pGV001 DNA (Volckaert et al., in preparation) with the restriction enzymes *Hae*III and *Hin*fI. The sample was treated with phenol and the DNA was precipitated with ethanol. Subsequently the fragments were radiolabelled by elongation of the 3′ recessed *Hin*fI-ends with DNA polymerase I (Klenow fragment A) in the presence of dGTP and [α-^{32}P]dATP [11]. Those fragments generated by one *Hae*III and one *Hin*fI cleavage are single-end labelled by this procedure.

The labelled digest was fractionated by electrophoresis on a 5% polyacrylamide gel in 0.04 M Tris–0.02 M sodium acetate (pH 7.8). The labelled fragments were located by autoradiography, excised and eluted by gently shaking in 1 M NaCl at room temperature for at least 1 h. The supernatant was filtered on Sephadex G-50, mixed with an equal volume of isopropanol and chilled at −20°C for 30 min to precipitate the DNA. The DNA was subsequently pelleted, washed with ethanol, dried, redissolved in water and stored at −20°C. Fragments labelled at one end only were selected for further experiments.

2.3. *Electrolytic reduction*

Controlled potential electrolysis was performed using a 3 electrode configuration (Tacussel Générateur Coulométrique type GCU) involving a mercury pool as working electrode (cathode), a platinum wire counter electrode and a saturated calomel reference electode (SCE). Reoxidation of the generated reduction products was prevented by separating the counter electrode from the working solution with a sintered glass disc.

The cathodic compartment contained 1 ml of a solution of nitroimidazole in 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0). To this solution 100 μl end-labelled DNA was added. The anodic compartment contained 15 mM NaCl and 1.5 mM sodium citrate (pH 7.0). Since oxygen prevents nitro-reduction [1] the solution was purged with N_2 to remove all traces of oxygen.

The reductive activation of the nitroimidazoles was carried out at a constant potential of −900 mV *vs* SCE. Control samples with DNA fragment in the absence of nitroimidazoles were included in all experiments to ascertain the absence of DNA degradation by electrolysis [12,13].

2.4. *Analysis of the DNA*

After electrolytic reduction the content of the reaction vessel was dispensed into two Eppendorf tubes (500 μl each). 50 μl of 3 M sodium acetate and 550 μl isopropanol were added and chilled at −20°C for 1 h. Subsequently, the DNA was pelleted, washed with 80% ethanol and redissolved in 150 μl of 0.3 M NaOAc (pH 7.2). The DNA was reprecipitated with 2.5 vols ethanol at −70°C for 15 min. One of the samples was treated with piperidine and evaporated to dryness as described for chemical DNA sequencing [14,15]. In parallel, a set of base-specific chemical degradation reactions was carried out as in [14,15]. All prepared samples were loaded in adjacent slots on a thin 8% sequencing gel [16].

After electrophoresis the DNA was fixed by immersing the gel for 15 min in a solution of 10% acetic acid. The gel was subsequently dried and exposed at ambient temperature to a Kodak X-omat AR film.

3. RESULTS AND DISCUSSION

In order to reveal the specificity of reaction of reduced nitroimidazoles with DNA, we treated single-end-labelled DNA fragments with the drug and ran the reaction products in adjacent lanes on sequencing gels. This approach has previously been used successfully to demonstrate the interaction of the antitumour antibiotics bleomycin and neocarzinostatin with DNA [9,10]. We performed experiments with different nitroimidazoles. Reduction of the nitro group is a prerequisite for the biological activity of the drug. Accordingly, we did not detect any degradation by exposing of the DNA to the parent drug. After treatment with reductively activated drug the DNA backbone is still largely intact, although sometimes slight degradation is found (fig.1, lanes, 2a,3a). Extensive degradation, however, can be induced by piperidine, which displaces modified bases from the DNA and cleaves the chain at positions rendered vulnerable by modification (fig.1, lanes 2b,3b). Since the

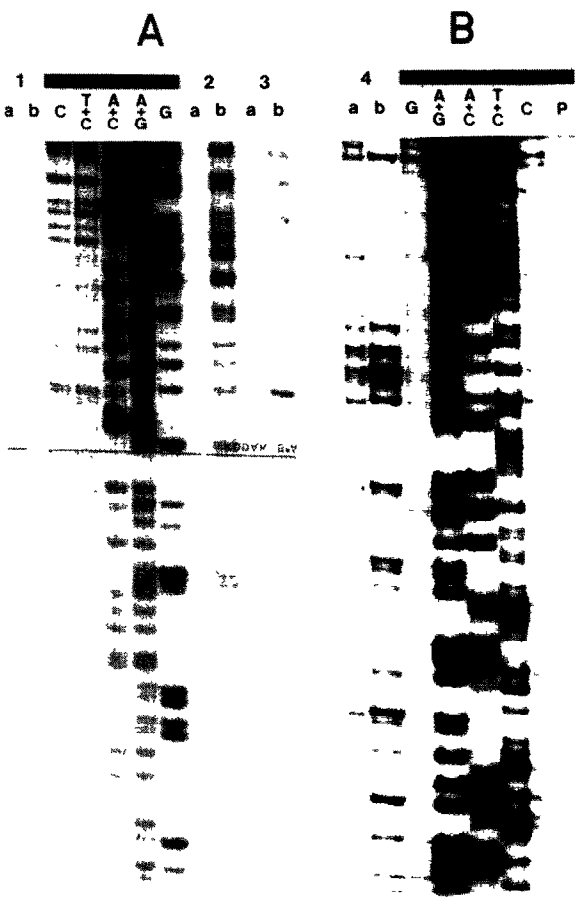

Fig.1. Determination of the base specificity of the interaction of reductively activated ornidazole with DNA. (A) Reductive activation in the presence of DNA; (B) addition of the DNA after electrolytic reduction of ornidazole. (1) 1×10^{-2} M $NaNO_2$, (2) 5×10^{-3} M ornidazole, (3) 5×10^{-4} M ornidazole, (4) 5×10^{-2} M ornidazole. Each sample is run before (a) and after (b) piperidine treatment. Sequencing degradations (G, AG, AC, TC, C, P) are shown below a heavy bar (P is a control sample treated with piperidine only). The top of the figure does not represent the position of the gel slots.

slight degradation observed in the absence of piperidine treatment is at identical positions to those induced by piperidine, we assume that the former scissions occur during the manipulation steps prior to gel electrophoresis (e.g. during boiling of the samples before loading the gel). Exposure of the DNA fragment to electrolysis in the presence of $NaNO_2$, but in the absence of the drug, does not lead to degradation (fig.1, lanes 1a,1b). This shows that the reactive intermediate is not the nitrite ion.

Addition of the DNA to the activated drug, i.e. after electrolytic reduction, yields an identical degradation pattern (fig.1, lanes 4a,4b). This indicates that the reactive intermediate is relatively stable in the absence of DNA.

Fig.1 clearly shows that degradation of the DNA fragment occurs almost exclusively at guanine residues. There is no sequence specificity. Minor cleavages occur at some, but not all, T residues. It should be stressed that only modifications can be observed which are prone to displacement by piperidine. The nature of the G modification remains to be determined, as well as the in vivo mechanism(s) by which this modification may be responsible for the mutagenic and radiosensitizing activities of the drug. The cleavage pattern shown in fig.1 after treatment with ornidazole is identical with those obtained with other nitroimidazoles, e.g. dimetridazole, ronidazole, MK-910, carnidazole and nimorazole (not shown).

All investigations previously described were based mainly upon changes of the physicochemical properties of the DNA or of DNA-containing solutions, such as viscometry, spectrophotometry, determination of melting temperature and renaturation profiles, determination of the single strand breaks by standard methods such as sucrose gradient ultracentrifugation. In contrast to our results, these experiments revealed fragmentation of DNA by activated nitroimidazoles [6–8]. Such fragmentation, however, might be restricted to the use of macromolecular DNA by these authors. We cannot exclude sequence specificity in this case. In addition, a preferential release of thymidine phosphates after electrolytic reduction of misonidazole [1-(3-methoxy-2-hydroxypropyl)-2-nitroimidazole] in the presence of DNA was observed in [8].

Our results are in agreement with the findings of authors in [5] who showed that the interaction of chemically reduced nitroimidazoles with DNA is directly proportional to the G + C content. This was also confirmed recently by authors in [17] who described the modification of guanine by chemically reduced 2-nitroimidazoles.

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