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DNA strand breakage by peroxidase-activated mitoxantrone

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Abstract—Spectroscopic evidence demonstrates that the alkylaminoanthraquinone mitoxantrone is a substrate for horseradish peroxidase in the presence of hydrogen peroxide and that the result of this interaction is the formation of an air-stable mitoxantrone-derived free radical. The mitoxantrone-derived free radicals or their further oxidation products appear to extensively cross-link with plasmid DNA by a reaction that is mitoxantrone concentration-dependent. Oxidative activation of mitoxantrone to a DNA-damaging species may contribute to the mechanism of action of this antitumour agent.

Peroxidase-mediated reactive intermediate formation has been implicated in the toxicity of a wide range of xenobiotics (Mason & Chignell 1982; Meunier 1987) including arylamine carcinogens (Flammang et al 1989). Mitoxantrone is an alkylaminoanthraquinone antitumour agent used clinically against advanced breast cancer and acute myeloid leukaemia (Koeller & Eble 1988). Experimental studies have shown that mitoxantrone binds to DNA, and also causes DNA single, double and protein-associated strand breaks (Cheng & Zee-Cheng 1983). These effects on DNA have been associated with inhibition of topoisomerase II by mitoxantrone (Tewey et al 1984). However, mitoxantrone free radical formation has been reported (Basra et al 1985) which could also result in damage to DNA. We report here free radical generation and DNA damage by mitoxantrone in the presence of horseradish peroxidase (HRP).

Materials and methods

Mitoxantrone dihydrochloride was a generous gift from Cyanamid G.B., Gosport, Hants, UK, and was reconstituted in water and stored at 4°C in the dark. Horseradish peroxidase E.C.1.11.1.7 (type XI, 500 units mg^{-1}) and all reagents were purchased from either Sigma Chemical Co., London or BDH, Poole, Dorset, UK.

Spectral studies on peroxidase Compound II formation. Spectral studies on the interaction of mitoxantrone with HRP Compound I following the addition of H_2O_2 (1·2 μM) to HRP (0·72 μM) were carried out at 37°C as described previously (d'Arcy Doherty et al 1986). A Perkin Elmer 552 dual beam spectrophotometer was used for all analyses with an identical concentration of mitoxantrone in the sample and reference cuvettes.

Electron spin resonance spectrometry. Electron spin resonance studies were carried out using a Varian E109 X-band spectrometer fitted with a signal accumulator and at a microwave frequency of 9·5 GHz. Incubation mixtures (1 mL) consisted of mitoxantrone (3 μmol), horseradish peroxidase (19 units), H_2O_2 (2·25 μmol) and phosphate buffer (pH 7·4, 100 μmol).

DNA strand breakage. Mitoxantrone (5–50 μM) was incubated with pBR322 plasmid DNA (0·3 μg) in the presence of HRP (0·012 units), H_2O_2 (50 nM) and Tris-HCl buffer 100 μM pH 8·0 in a final volume of 100 μL. The reaction was initiated by the addition of plasmid DNA to the other components, the reactants were then centrifuged for a few seconds to ensure thorough mixing and incubated in the dark at 37°C for 30 min. Each reaction was terminated by the addition of NaCl (5·0 M, 100 μL) to facilitate extraction. The drugs were extracted with water-saturated butanol (200 μL, 3–5 extractions) until no colour could be observed in the aqueous phase. The DNA was precipitated by the addition of 400 μL ethanol (−20°C) and left for 1 h at −20°C. The samples were then centrifuged for 10 min at 12000 *g* in a microfuge to pellet the DNA and the ethanol supernatant was discarded. Residual ethanol was removed using a glass capillary followed by placing the samples under vacuum for 5 min. The DNA was then reconstituted with 5 μL Tris-EDTA buffer pH 8·0, centrifuging (3 s) to ensure thorough mixing and incubation at 37°C for 15 min to facilitate dissolution. Drug-mediated DNA single strand and double strand breaks were identified by separating the supercoiled plasmid DNA (Form I) from open circular (Form II) and linear (Form III) using submerged agarose gel (1%) electrophoresis at 150V, for 1 h in Tris base (24·2%)/glacial acetic acid (5·7%)/EDTA (0·05 M) buffer. DNA was visualized by staining with ethidium bromide (2 $\mu g\ mL^{-1}$) and transilluminating under UV light (300 nm) and the gels photographed. The negatives were scanned using a laser densitometer (LKB 2202) linked to an Apple II microcomputer system and LKB "Gel Scan" dedicated software for data capture and analysis of non-Gaussian peaks.

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Results

The involvement of mitoxantrone as an electron donor for conversion of Compound I (FeV) to HRP (FeIII) via Compound II was investigated. The interconversion of these different oxidation states of HRP is well known to be associated with characteristic spectral shifts (Eichhorn 1973). Fig. 1 shows that addition of H_2O_2 to HRP produced a bathochromic and hypochromic shift from the absorbance maximum of HRP (FeIII) at 403 nm to that characteristic maximum of compound I (410 nm). Compound I was unstable and slowly converted to compound II (λ_{max} 418 nm). However, the rate of this conversion was shown to be accelerated in the presence of stoichiometric amounts of mitoxantrone. Addition of an excess of mitoxantrone (2·4 μM) resulted in the complete regeneration of HRP (FeIII). These results indicate one and/or two electron oxidation of mitoxantrone by HRP. By analogy with the action of HRP on other substrates (Mason & Chignell 1982; d'Arcy Doherty et al 1986) formation of a mitoxantrone free radical was likely to occur.

Electron spin resonance (esr) spectrometry was used to investigate mitoxantrone free radical generation. Fig. 2 shows that when mitoxantrone was incubated with HRP and H_2O_2 an esr signal was observed. The esr spectrum was a broad envelope with a peak-to-peak line width of 20 G and no hyperfine splitting. No spectrum was observed unless HRP, H_2O_2 and mitoxantrone were all present. Attempts to resolve the spectrum into a hyperfine pattern by reducing the modulation amplitude and increasing the scan time in combination with computer enhancement were unsuccessful. The possible involvement of peroxidase-generated mitoxantrone free radical in DNA damage was subsequently investigated using pBR322 plasmid DNA as a model system. Plasmid DNA is supercoiled (Form I) but when a single strand break occurs it assumes an open circular (Form II) configuration. A coincidental single strand break on the complementary strand or a direct double strand break

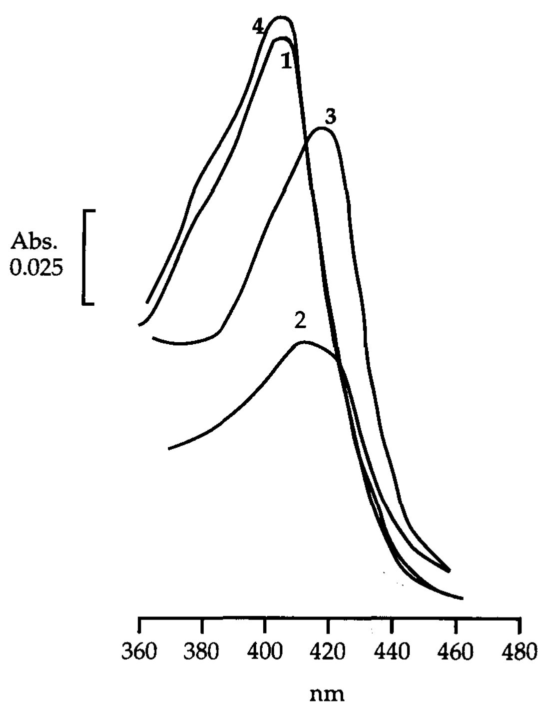

FIG. 1. Spectral changes observed following the addition of H_2O_2 and mitoxantrone to horseradish peroxidase. The spectrum of ferric HRP (0·72 μM) was recorded (1) before and (2) after the addition of H_2O_2 (1·2 μM). Upon addition of mitoxantrone (0·24 μM), compound II (3) was observed. Following the addition of a stoichiometric excess of mitoxantrone (2·25 μM) the spectrum of ferric HRP (4) was regenerated. The spectra observed are typical of 3 experiments.

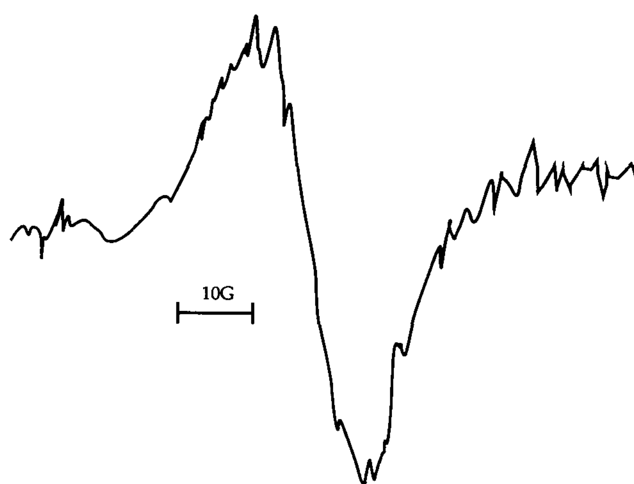

FIG 2. Electron spin resonance spectrum generated in a system containing mitoxantrone (3·3 mM), HRP (19 units) and H_2O_2 (2·3 mM). Esr operating conditions were: microwave power 10 mW; modulation amplitude 0·5 G; time constant 1 s; scan time 2 min; scan range 100 G; receiver gain $8{\cdot}0 \times 10^3$. The spectrum was typical of 3 replicates.

produces linear DNA (Form III). The effect of incubating mitoxantrone on plasmid DNA in the presence of HRP and H_2O_2 at pH 7.4 is shown in Fig. 3. At 10 μM mitoxantrone a significant increase in DNA single strand breakage was observed whilst 25 μM mitoxantrone resulted in total loss of DNA Form I, II and III in the incubation mixture (Fig. 3). A blue colouration in the loading wells of lanes 10–12 was evident. Mitoxantrone at 50 μM, however, produced no marked loss of plasmid DNA. Control experiments show that neither mitoxantrone alone or combinations of mitoxantrone, HRP or H_2O_2 produced DNA damage (Fig. 4).

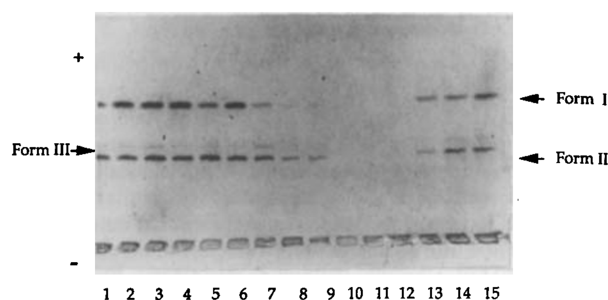

FIG. 3. Agarose gel electrophoresis of pBR322 plasmid DNA (300 ng) after incubating with HRP (0·012 units), H_2O_2 (0·05 μM) for 30 min at 37°C in the absence of mitoxantrone (lanes 1–3) and presence of 5 μM (lanes 4–6), 10 μM (lanes 7–9), 25 μM (lanes 10–12) and 50 μM (lanes 13–15) mitoxantrone. Form I = supercoiled DNA; Form II = open circular DNA; Form III = linear DNA.

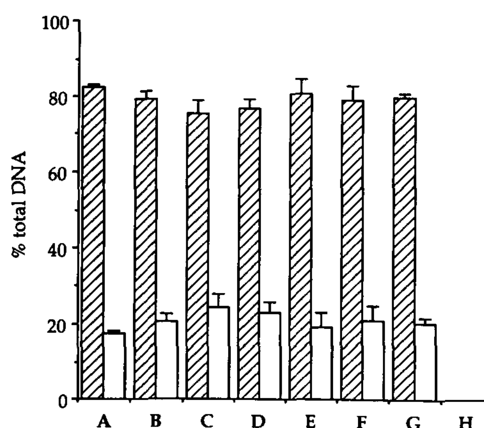

FIG. 4. Effect of mitoxantrone (MXT), HRP and H_2O_2 on pBR322 plasmid DNA. A = DNA alone; B = DNA + HRP; C = DNA + H_2O_2; D = DNA + HRP + H_2O_2; E = DNA + MXT; F = DNA + HRP + MXT; G = DNA + H_2O_2 + MXT; H = DNA + HRP + H_2O_2 + MXT. Hatched columns are supercoiled (Form I) DNA, open column are single strand break (Form II) DNA. Concentration of mitoxantrone was 25 μM, all others as given in Fig. 3. Results are the mean + s.d. of three determinations.

Discussion

This study presents spectral and esr evidence that mitoxantrone is a substrate for HRP and that the result of this interaction is the formation of a mitoxantrone-derived free radical. This is consistent with the oxidation of substrates by HRP and suggests

the formation of a one-electron oxidation product of mitoxantrone (see Fig. 5). The absence of hyperfine coupling in the esr spectrum suggests that HRP oxidation of mitoxantrone ultimately results in a polymeric free radical species. This high molecular mass polymer free radical would rotate slowly on the esr time scale leading to the esr line broadening observed (Fig. 2). In support of this, incubations of mitoxantrone with HRP and H_2O_2 led to the formation of a blue precipitate, insoluble in water and most organic solvents (results not given). The nature of this free radical is different to that observed previously where mitoxantrone was shown to be reductively metabolized by purified NADPH-dependent cytochrome P450 reductase (Sinha et al 1983) and human liver microsomes (Basra et al 1985). Under reductive conditions a mitoxantrone semiquinone is observed that can rapidly reduce oxygen and hence is not stable in air. The results of the present study show the presence of an air-stable radical and are in agreement with results of previous workers who also have shown that HRP/H_2O_2-dependent metabolism of mitoxantrone results in an electrophilic cation radical (Kolodziejcyk et al 1986). This was subsequently shown to be associated with one electron oxidation of the aromatic nitrogen atoms of mitoxantrone (Reszka et al 1988).

The mitoxantrone-derived free radicals or their further oxidation products generated in this study appeared to react with plasmid DNA. The result was the formation of a species of DNA that when subjected to agarose gel electrophoresis did not migrate or enter the gel but remained as a blue (mitoxantrone) coloured complex in the gel loading wells. This suggests extensive cross-linking of the mitoxantrone oxidation product with DNA. The lack of effect at a higher concentration of mitoxantrone (50 μM) (see Fig. 3) indicates that DNA interactions and polymer formation are competing pathways. Further studies are required to ascertain the exact nature of the oxidized mitoxantrone/DNA product. It is possible that the cation radical of mitoxantrone and/or the unstable di-imino-mitoxantrone derivative that has been shown to result from HRP/H_2O_2 oxidation of mitoxantrone (Reska et al 1988) are involved in this cross linkage. Other diamino compounds have been shown to cross-link DNA in the presence of HRP/H_2O_2 (O'Brien 1985) and prevent plasmid DNA migration (O'Brien et al 1985). In contrast to these results for mitoxantrone no free radical spectra were observed when either doxorubicin or the cytotoxic anthrapyrazole C1941 (5-[(aminoalkyl)amino]anthra[1,9-cd]pyrazol-6-(2H)-one) were incubated under identical conditions with HRP and H_2O_2 and nor did these compounds affect supercoiled plasmid DNA (results not shown). This indicates the importance of the *p*-diamino arrangement of mitoxantrone in this route of metabolism. Recently iminodaunorubicin, a quinoneimine, has also been shown to undergo peroxidase-mediated cation radical formation (Reska et al 1989). Fig. 5 summarizes the involvement of HRP in mitoxantrone activation to a reactive species.

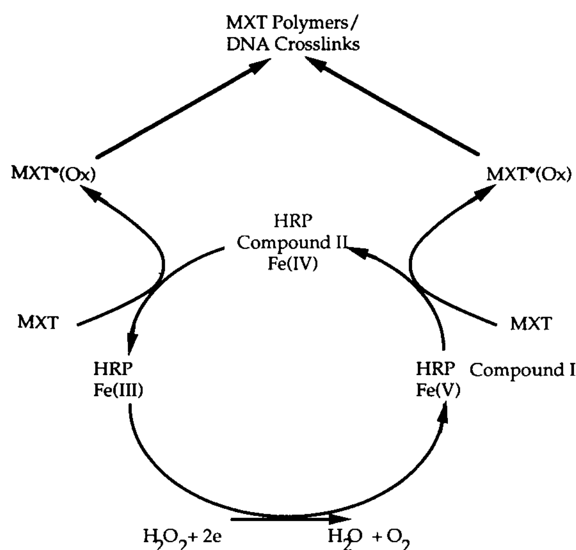

FIG. 5. Scheme for one-electron oxidation of mitoxantrone (MXT) by horseradish peroxidase (HRP).

Although reductive activation of mitoxantrone (and the structurally similar ametantrone) to a semiquinone free radical has been reported (Basra et al 1985; Sinha et al 1983) the levels generated do not appear to result in damage to lipids (Patterson et al 1983; Kharasch & Novak 1985) or DNA (Fisher & Patterson unpublished). In contrast, the occurrence of peroxidase-mediated mitoxantrone free radical formation at physiological pH suggests a mitoxantrone reactive intermediate may be involved in the cellular DNA strand breakage (Cheng & Zee-Cheng 1983) and nucleic acid condensation (Kapuscinski & Darzynkiewicz 1986) observed for this compound. In this respect prostaglandin endoperoxidase may be a candidate as an oxidative activater of mitoxantrone intracellularly since arylamines are known substrates for this peroxidase (Eling et al 1983; Flammang et al 1989) and it has been detected in a variety of tumour cell types (Hubbard et al 1988).

References

Basra, J., Wolf, C. R., Brown, J. R., Patterson, L. H. (1985) Evidence for human liver mediated free radical formation by doxorubicin and mitoxantrone. Anti-Cancer Drug Design 1: 45–52

Cheng, C. C., Zee-Cheng, R. K. Y. (1983) The design, synthesis and development of a new class of potent antineoplastic anthraquinones. Prog. Med. Chem. 20: 83–117

d'Arcy Doherty, M., Wilson, I., Wardman, P., Basra, J., Patterson, L. H., Cohen, G.M. (1986) Peroxidase activation of 1-naphthol to naphthoxy or naphthoxy-derived radicals and their interaction with glutathione. Chem-Biol. Interact. 58: 199–215

Eichhorn, G. L. (1973) Peroxidases and catalases. In: Eichhorn, G.L. (ed.) Inorganic Biochemistry, Vol. 2, Elsevier, Amsterdam, pp 988–1021

Eling, T., Boyd, J., Reed, G., Mason, R., Sivarajah, K. (1983) Xenobiotic metabolism by prostaglandin endoperoxide synthetase. Drug Metab. Revs. 14: 1023–1053

Flammang, J. T., Yamazoe, Y., Benson, R. W., Roberts, D. W. et al (1989) Arachidonic acid-dependent peroxidative activation of carcinogenic arylamines by extrahepatic human tissue microsomes. Cancer Res. 49: 1977–1982.

Hubbard, W. C., Alley, M. C., McLemore, T. L., Boyd, M. R. (1988) Profiles of prostaglandin biosynthesis in sixteen established cell lines derived from human lung, colon, prostate and ovarian tumours. Ibid. 48: 4770–4775

Kapuscinski, J., Darzynkiewicz, Z. (1986) Relationship between the pharmacological activity of antitumour drugs ametantrone and mitoxantrone (Novantrone) and their ability to condense nucleic acids. Proc. Natl. Acad. Sci. (USA) 83: 6302–6306

Kharasch, E. D., Novak, R. F. (1985) Mitoxantrone and ametantrone inhibit hydroperoxide-dependent initiation and propagation reactions in fatty acid peroxidation. J. Biol. Chem. 260: 500–506.

Koeller, J., Eble, M. (1988) Mitoxantrone: a novel anthracycline derivative. Clinical Pharmacy 7: 574–581

Kolodziejcyk, P., Reszka, K., Lown, J. W. (1986) Horseradish peroxidase oxidation of mitoxantrone. Spectrophotometric and electron paramagnetic resonance studies. J. Free Rad. Biol. Med. 2: 25–32

Mason, R. P., Chignell, C. F. (1982) Free radicals in pharmacology and toxicology-selected topics. Pharmacol. Revs. 33: 189–211

Meunier, B. (1987) Horseradish peroxidase: a useful tool for

modelling the extra-hepatic biooxidation of exogens. Biochimie 69: 3–9

O'Brien, P. J. (1985) Free radical mediated DNA binding. Environmental Health Perspectives 64: 219–232

O'Brien, P. J., Gregory, B., Fourney, B., Davison, W., Rahimtula, A.D., Tsuruta, Y. (1985) One-electron oxidation in cell toxicity. In: Boobis, A. R., Caldwell, J., Matteis, F., Elcombe, C. R. (eds) Microsomes and Drug Oxidations, Ellis Horwood, London, pp 284–295

Patterson, L. H., Gandecha, B. M., Brown, J. R. (1983) Ametantrone, an anthraquinone antitumour agent that does not cause lipid peroxidation in vivo. Comparison with daunorubicin. Biochem. Biophys Res. Commun. 110: 399–405

Reszka, K., Kolodziejczyk, P., Lown, J. W. (1988) Enzymatic oxidative activation and transformation of the antitumour agent mitoxantrone. J. Free Rad. Biol. Med. 5: 13–2

Reszka, K., Kolodziejcyk, P., Lown, J. W. (1989) Enzymatic oxidation of 5-iminodaunorubicin. Spectrophotometric and electron paramagnetic resonance studies. Biochem. Pharmacol. 38: 803–809

Sinha, B.K., Motten, A.G., Hanck, K.W. (1983) The electrochemical reduction of mitoxantrone and daunomycin: biochemical significance in superoxide formation. Chem.-Biol. Interact. 43: 371–373

Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D., Lui, L.F. (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466–468

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New heteroaryl derivatives of fentanyl

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Abstract—The preparation of analogues of fentanyl with *N*-phenyl replaced with a heterocyclic aromatic ring, and with *N*-alkyl/arylalkyl *N*-acyl substituents is reported. Only those compounds carrying an *N*-phenylethyl substituent were active in the rat tail-withdrawal test. Fentanyl (1) is the prototype of the 4-anilidopiperidine class of opioid analgesics (Casy & Parfitt 1986). Several recent publications (Casy & Huckstep 1988; Bagley et al 1989) on this series have dealt with heterocyclic and aromatic modifications of the fentanyl molecule. Although the antinociceptive potency of most novel derivatives was greater than that of morphine, they had reduced activity when compared with fentanyl itself. To acquire further information on the structure-reactivity relationships of the series, new heteroaryl derivatives have been prepared and tested for antinociceptive activity.

Chemistry

The intermediate 4-(heteroanilido)-piperidines were obtained by treating the 4-amino-*N*-substituted piperidine with the appropriate heterocyclic halide. Acylation of these products with the corresponding acid chloride in the presence of triethylamine (Lobezoo et al 1980) yielded the required fentanyl analogues 2–13 (Table 1). Infra-red spectroscopy, ^{1}H-NMR spectroscopy and elemental analysis confirmed the structures shown.

Preparative work

Melting points are uncorrected. Spectroscopic data (IR, ^{1}H-NMR) support structures in all cases. Microanalyses were performed on a Perkin-Elmer 240 by CSIC laboratories (Madrid Spain).

5-Bromo and 8-bromoquinolines were prepared as described by Butler & Gordon (1975), 5-bromoisoquinoline as described by Gordon & Pearson (1964), 9-chloroacridine as described by Atwell et al (1984), and 7-chlorobenzo[b][1,8] phenanthroline as described by Elslager & Tendrick (1962). 1-Methyl-4-aminopiperidine and 1-phenylethyl-4-aminopiperidine were prepared by reduction of the corresponding 4-piperidone oximes with lithium aluminium hydride by the standard procedure (Harper & Chignell 1964).

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4-Heteroanilido-piperidines (general procedure). A mixture of 4-aminopiperidine-*N*-substituted (40 mmol), heteroaryl halide (20 mmol), copper powder (20 mmol) and triethylamine (5 mL) in 1-pentanol (30 mL) was stirred at the reflux temperature for 4 h. The mixture was concentrated in-vacuo and the solid was added to 10% HCl (50 mL). The suspension was stirred and the melt was poured slowly into an excess of ice and concentrated ammonium hydroxide (150 mL) and extracted with ether (4 × 100 mL). The organic layer was washed with water and dried over anhydrous sodium sulphate. The crude products were purified by column chromatography and recrystallized.

4-Heteroanilido-4-acyl-piperidines (2–13) (general procedure). To a mixture of 4-heteroanilidopiperidine (20 mmol) in chloroform (30 mL) and triethylamine (5 mL) was added dropwise a solution of the acid chloride (30 mL) in chloroform (5 mL). The reaction mixture was stirred at reflux temperature for 1 h. The mixture was concentrated in-vacuo and the residue was extracted with 10% HCl–ether (1 : 1) (50 mL). The aqueous layer was alkalinized with concentrated ammonium hydroxide (100 mL) and extracted with chloroform (4 × 100 mL), washed with water and dried over anhydrous sodium sulphate. The products were purified by recrystallization.

Pharmacology and discussion

The antinociceptive activities of fentanyl and its analogues were assessed in rats by the tail-withdrawal test (Janssen et al 1963, Table 1). When the *N*-phenylethyl and *N*-propionyl substituents were present (compounds 2-5), the antinociceptive activity decreased while a complete lack of activity at 2·5 mg kg^{-1} was observed for compound 6 with an increased steric bulk (Series 5A). The *N*-heteroaryl analogues have potencies greater than that of morphine, with significant differences between 5- and 8-quinolyl isomers (2,3) indicating that the heterocyclic conforma-