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DNA BASE DAMAGE BY β-LACTAM, TETRACYCLINE, BACITRACIN AND RIFAMYCIN ANTIBACTERIAL ANTIBIOTICS

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Abstract—Several antibacterial antibiotics have been shown to participate with transition metal ions in chemical reactions leading to the formation of reactive oxygen species. An important host defence mechanism for dealing with invading bacteria involves the production of reactive oxygen species, such as superoxide, hydrogen peroxide and hypochlorous acid, by phagocytic cells. The production of reactive oxygens by redox cycling antibacterial antibiotics has led us to suggest that a 'phagomimetic' contribution may also be made *in vivo*. Here we show that four structurally different antibacterial antibiotics, in the presence of added copper salt, bring about oxidative modification to bases in DNA detected using gas chromatography–mass spectrometry. The drug most damaging to DNA was rifamycin SV which was more active than a reference mixture of hydrogen peroxide and ascorbic acid.

Antibiotics are produced to specifically damage vital components of invading micro-organisms or host cells. We can conveniently divide antibiotics into three main groups depending on their cell targets: antifungal antibiotics are lipophilic and bind to sterol molecules in eukaryotic cell membranes, antitumour antibiotics bind or intercalate host cell DNA, whereas the major group of antibiotics are selected for their antibacterial properties. The mechanisms by which antibiotics of the latter group kill or inhibit the growth of invading bacteria are thought to involve: interference with key bacterial cell enzymes, impairment of protein synthesis and damage to membranes. In addition, most antibiotics have a variety of poorly characterized pharmacological properties which contribute to their overall efficacy *in vivo*. Exploring alternative mechanisms by which diverse antibiotics can damage cell components, the authors and others [1, 2] have proposed that a common feature of many antibacterial antibiotics is their ability to undergo redox cycling and generate oxygen radicals (phagomimetic activity) since this could explain some of their antibacterial properties *in vivo*. The ability of activated phagocytic cells to produce and utilize oxygen radicals as part of microbial killing in the body is now a well established observation [3].

Previous work has shown that β-lactams, tetracyclines, bacitracins and rifamycins (Fig. 1) can degrade DNA in the presence of metal ions [1, 4–6]. Here, we extend our studies to examine damage to DNA at the molecular level using gas chromatography–mass spectrometry (GC–MS) [7–9]. Hydroxyl radical damage to DNA has been shown to produce characteristic "footprints" of base modification and the patterns of such base modification reflect site-specific binding of iron and copper ions to DNA and chromatin [10, 11]. Using copper, ascorbic acid and hydrogen peroxide as an established reference model for demonstrating hydroxyl radical damage to DNA [10–12] we show here that several antibacterial antibiotics in the presence of copper ions are able to modify bases in DNA.

MATERIALS AND METHODS

Methicillin (sodium salt), bacitracin, thymine, 6-azathymine, cytosine, adenine, guanine, isobarbituric acid (5-hydroxyuracil), 5-methylcytosine, DNA (herring testes) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from the Sigma Chemical Co. (Poole, U.K.). Doxycycline HCl was a gift from Cox and Company and rifamycin SV was the WHO 1st International Reference Preparation with an assigned potency value of 887 I.U. per mg. All other chemicals were of the highest grades available from BDH Chemicals (Poole, U.K.).

Damage to DNA by antibiotics. The following reagents were added to new clean glass tubes: 0.8 mL of DNA 2 mg/mL dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The buffers were prepared with double glass-distilled water known to be metal ion free. Antibiotic solution (0.4 mL; 5 mM), dissolved in double distilled water, was followed by 0.4 mL of 2 mM cupric chloride. Distilled water was added to controls and blanks to standardize volumes. The reference standard for hydroxyl radical generation (100% damage under the conditions described) consisted of 0.2 mL of 400 mM hydrogen peroxide and 0.2 mL of 2 mM ascorbate added instead of the antibiotics in the presence of Cu^{2+} ions.

Reaction mixtures were incubated at 37° for 3 hr in a shaking waterbath. Incubated samples were sealed in dialysis tubing and dialysed against 4 L of double-distilled water for 18 hr at 4°. After this time, with constant stirring, the contents of the dialysis

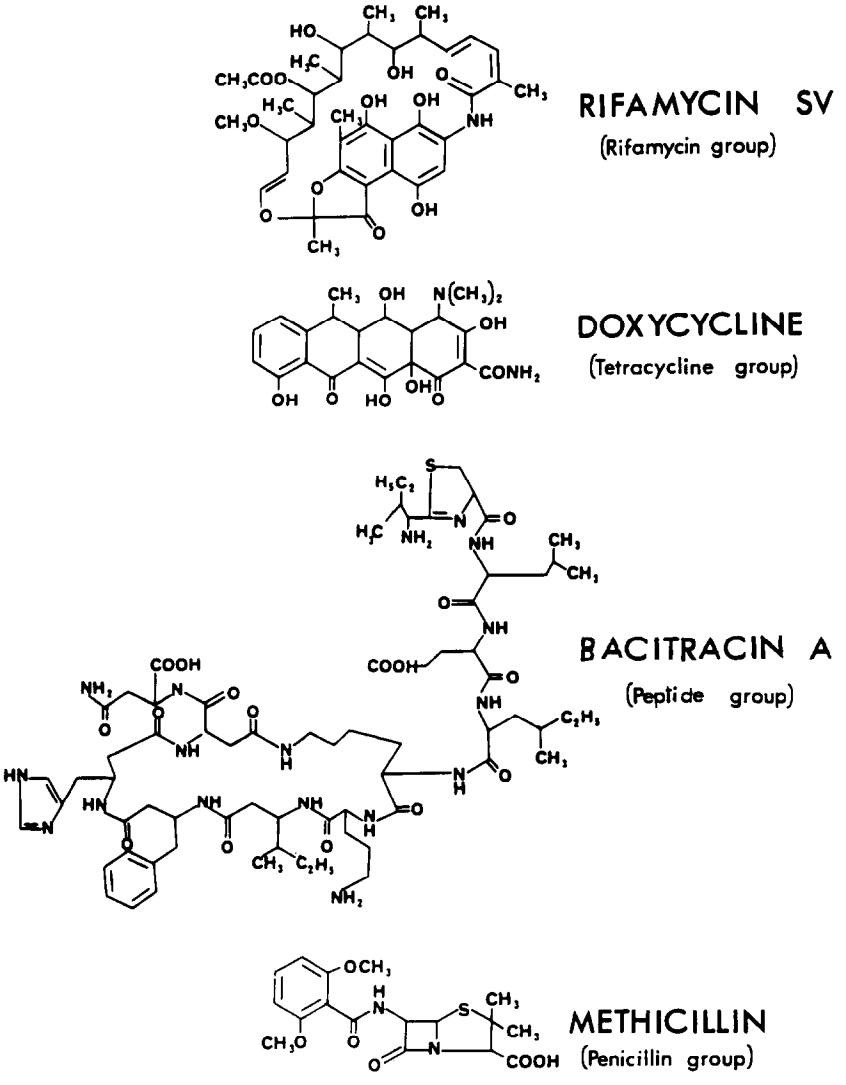

Fig. 1.

tubing were transferred to hydrolysis tubes and freeze-dried.

Hydrolysis of DNA and derivatization of bases [6]. 6-Azathymine (10 μg) was added as an internal standard, to control variations in sample injection, followed by 0.9 mL of concentrated formic acid (98%). Air was evacuated from the tube before sealing and heating at 130° for 30 min. When cool, solutions were again freeze-dried. Bases were derivatized by adding 0.2 mL of BSTFA : acetonitrile (1:1), sealing the tubes and heating for 20 min at 120°. When cool, the derivatized bases were stored in sealed vials at −20° until required.

Gas chromatography–mass spectrometry [7]. One microlitre of sample was injected, and split 12:1, into a Perkin–Elmer 8420 capillary gas chromatograph with the injection point held at 250°. The chromatograph was coupled to a Perkin–Elmer Ion Trap detector. The capillary column was a wall coated open tubular fused silica, 25 M × 0.25 mm column coated with 5% phenylmethyl silicone, cross linked and bonded (Chrompack, CP Sil 8 CB). The oven temperature was held at 100° for 1 min then increased 10°/min to 250°. Data was collected between 6 and 19 min. The mass range was 100 amu to 480 amu and gas flow 18 psi. The total ion chromatograms obtained were individually analysed for selective ions by ion extraction.

RESULTS

The individual bases of thymine, 6-azathymine, cytosine, 5-methylcytosine, adenine and guanine were identified by the use of authentic material and their mass spectra using characteristic molecular ions $(M)^+$ and their base peaks, which usually correspond to the $(M\text{-}CH_3)^+$ ion. When total ion chromatograms of controls and antibiotic and standard test samples (hydrolysed and derivatized) were compared, the presence of up to five additional peaks in the test samples could be recognized. These are shown as peaks 5, 7, 8, 9 and 11 in Fig. 2. Based on

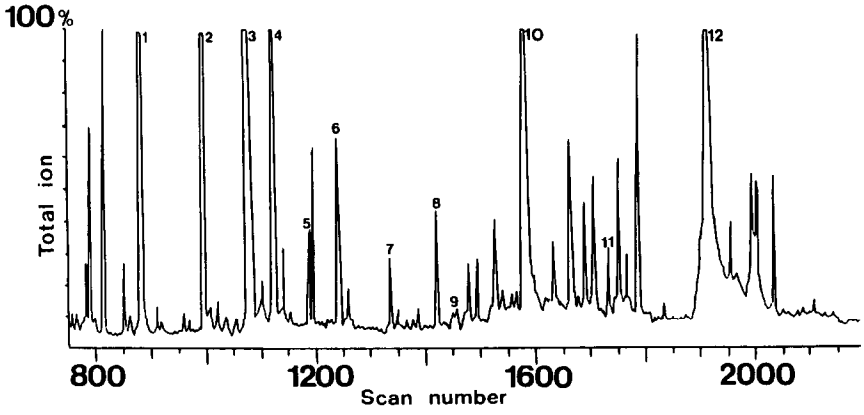

Fig. 2. A total ion chromatogram of DNA bases after treatment with rifamycin SV and copper salt. Key showing the ions used to identify peaks:

	Characteristic/base peaks	Molecular/significant ions
1. Thymine	255	270
2. 6-Aza-thymine	256	271
3. Cytosine	240	255
4. Methylcytosine	254	269
5. 5-Hydroxyuracil	329	344
6. Cytosine	312	327
7. 5-Hydroxycytosine	328	343
8. *cis*-Thymine glycol	259	433
9. *trans*-Thymine glycol	259	433
10. Adenine	264	279
11. 8-Hydroxyadenine	352	367
12. Guanine	352	367

Except for peaks 8 and 9 the base peaks corresponded to the m/z $(M\text{-}CH_3)^+$ ion. Total ion chromatograms of DNA, DNA + Cu^{2+}, DNA + H_2O_2 and DNA + antibiotics alone were as in Fig. 2 minus peaks 5, 7, 8, 9 and 11.

previous assignments for DNA bases [7, 13] and chromatographic positions, the following identifications were made: peak 5 was characteristic of 5-hydroxyuracil and peak 7 as 5-hydroxycytosine. Both 5-hydroxyuracil and 5-hydroxycytosine are formed by deamination and/or dehydration of cytosine [14]. Peaks 8 and 9 were characteristic of *cis*- and *trans*-thymine glycol. Between peaks 8 and 9 a small peak, identified as 5,6-dihydroxyuracil, was present but only in the copper–H_2O_2–ascorbate standard [9] and in the DNA incubated with rifamycin SV and copper. Peak 11 was identified as 8-hydroxyadenine.

Quantitative results shown in Fig. 3 for the DNA bases, modified by the different antibiotics, were calculated as peak areas, and expressed as a percentage of the control standard hydroxyl radical generating system (i.e. the copper salt with H_2O_2 and ascorbate). 6-Azathymine was used as an internal standard to compensate for injection variations. From Fig. 3 it can be seen that rifamycin SV is considerably more effective than the reference hydroxyl radical generating system in damaging DNA bases and forming 5-hydroxycytosine and thymine glycol. In the absence of added copper ions rifamycin SV was still able to damage DNA suggesting that some copper may be associated with the antibiotic preparation or with DNA.

Doxycycline and methicillin brought about similar patterns of damage to DNA as did rifamycin SV, although they were considerably less effective. Bacitracin induced only minor, if at all detectable, damage to DNA bases in agreement with previously observed release of trace amounts of malondialdehyde from deoxyribose, the sugar moiety of DNA [5].

DISCUSSION

The antibiotics methicillin, doxycycline, bacitracin and rifamycin SV are antibacterial agents representing distinct classes of antibiotic with no structural similarities (Fig. 1). Their biological activities towards bacterial cells are thought to involve methicillin inhibiting key enzymes so preventing transpeptidation between peptidoglycans, doxycycline inhibiting translation by impairing protein synthesis through binding to the 30s ribosomal sub-units, bacitracin inhibiting the incorporation of amino acids into the cell wall mucopeptide and rifamycin SV inhibiting DNA-dependent RNA synthesis. In addition, these antibiotics have been shown to undergo oxidation which in the presence of transition metal ions such as iron and copper, leads to the formation of highly reactive and

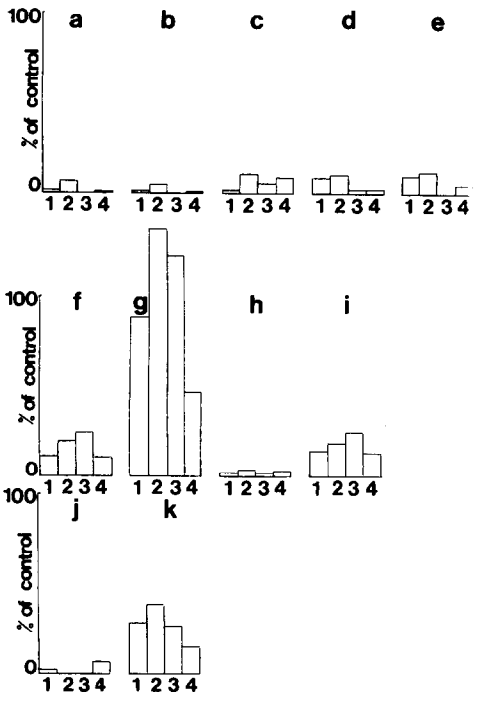

Fig. 3. Quantitation was performed on the characteristic base peak in each case. Peak areas corresponding to *cis*- and *trans*-thymine glycol were summed and represented as a single area. Two cytosine peaks are seen as two TMS derivatives are formed, one as an additional TMS group on the nitrogen. Key: a, DNA; b, DNA + Cu^{2+}; c, DNA + H_2O_2; d, DNA + bacitracin; e, DNA + bacitracin + Cu^{2+}; f, DNA + rifamycin SV; g, DNA + rifamycin SV+ Cu^{2+}; h, DNA + doxycycline; i, DNA + doxycycline + Cu^{2+}; j, DNA + methicillin; k, DNA + methicillin + Cu^{2+}. Ions: 1, 329; 2, 328; 3, 259; 4, 352.

damaging oxygen species such as the hydroxyl radical ($^{\cdot}OH$).

The precise chemistry leading to hydroxyl radical formation, for the different antibiotics, has not yet been established but can be summarized as the following simple reactions: the antibiotic in its reduced form (ABH_2) undergoes oxidation with a transfer of electrons to either molecular oxygen to form superoxide (O_2^-) or to transition metal ions such as copper (e.g. Eqns 1 and 2). When an electron is transferred to copper it may subsequently be transferred to molecular oxygen, again forming superoxide. Superoxide can act both as a reductant for transition metal ions or as a precursor of hydrogen peroxide (H_2O_2) (Eqn 3). The final result is that Fenton chemistry, leading to $^{\cdot}OH$ radical formation, takes place by the reaction of reduced copper with hydrogen peroxide (Eqn 4).

$$ABH_2 \text{ (reduced)} + 2O_2 \rightarrow AB \text{ (oxidized)} + 2O_2^- + 2H^+ \quad (1)$$

$$ABH_2 \text{ (reduced)} + 2Cu^{2+} \rightarrow AB \text{ (oxidized)} + 2Cu^+ + 2H^+ \quad (2)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + {}^{\cdot}OH \quad (4)$$

The "footprinting" of hydroxyl radical damage to DNA, at the molecular level, has recently been possible through the application of GC–MS techniques pioneered by Dizdaroglu and his colleagues [7, 13, 15, 16]. Copper salts in the presence of suitable reductants such as ascorbate and hydrogen peroxide bring about substantial damage to DNA characteristic of the hydroxyl radical produced during ionizing radiation in aqueous solutions [13]. Using this defined technique of $^{\cdot}OH$ radical formation and damage to DNA as a reference, we have compared the ability of four structurally different antibiotics to substitute for ascorbate and hydrogen peroxide and modify bases in DNA. As suggested in Eqns 1–4, the antibiotics are able to react with a cupric salt and molecular oxygen to generate hydroxyl radicals. Copper-dependent damage to the bases in DNA, when ascorbate and hydrogen peroxide are present, has been shown to produce substantial modification of the guanine base resulting in the formation of 8-hydroxyguanine [10]. However, with the approach described here we were unable to detect 8-hydroxyguanine using our column and equipment. 8-Hydroxyguanine is known to be extremely labile [17] and requires stringent column specifications and operating conditions for separation and quantitation (M. Dizdaroglu, personal communication).

The tetracycline antibiotic doxycycline and the β-lactam antibiotic methicillin damage DNA bases in the presence of copper, confirming previously observed results showing changes in the electrophoretic mobility of DNA [1, 4]. Bacitracin has also been shown to weakly damage DNA by releasing thiobarbituric acid-reactive material from the deoxyribose sugar moiety of DNA [5]. Here, using GC-MS, evidence for bacitracin damaging DNA bases was poor, if at all detectable. By far the most reactive and DNA-damaging antibiotic was rifamycin SV, which produced more base damage than the standardized reference hydroxyl radical generating system of copper, ascorbate and hydrogen peroxide. In this aspect, it is interesting to note that rifamycin SV has been reported to possess antiviral properties [18].

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REFERENCES

1. Quinlan GJ and Gutteridge JMC, Oxidative damage to DNA and deoxyribose by β-lactam antibiotics in the presence of iron and copper salts. *Free Rad Res Commun* **5**: 149–158, 1988.
2. Ames JR, Ryan MD and Kovacic P, Mechanism of antibacterial action: electron transfer and oxy radicals. *J Free Rad Biol Med* **2**: 377–391, 1986.

3. Babior B, Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* **298**: 659–688, 1978.
4. Quinlan GJ and Gutteridge JMC, Hydroxyl radical generation by the tetracycline antibiotics with free radical damage to DNA, lipids and carbohydrate in the presence of iron and copper salts. *Free Rad Biol Med* **5**: 341–348, 1988.
5. Quinlan GJ and Gutteridge JMC, Bacitracin and Bacitracin-zinc complex damage DNA and carbohydrate in the presence of iron and copper salts. *Free Rad Res Commun* **7**: 37–44, 1989.
6. Quinlan GJ and Gutteridge JMC, Oxygen radical damage to DNA by Rifamycin SV and copper ions. *Biochem Pharmacol* **36**: 3629–3633, 1987.
7. Dizdaroglu M, Application of capillary gas chromatography–mass spectrometry to chemical characterisation of radiation-induced base damage to DNA: implications for assessing DNA repair processes. *Anal Biochem* **144**: 593–603, 1985.
8. Dizdaroglu M and Gajewski E, Selected-ion mass spectrometry: assays of oxidative DNA damage. In: *Methods of Enzymology* (Eds. Packer L and Glazer AN), Vol. 186, pp. 530–544. Academic Press, San Diego, 1990.
9. Dizdaroglu M, Chemical determination of free radical-induced damage to DNA. *Free Rad Biol Med* **10**: 225–242, 1991.
10. Aruoma OI, Halliwell B, Gajewski E and Dizdaroglu M, Copper-ion-dependent-damage to bases in DNA in the presence of hydrogen peroxide. *Biochem J* **273**: 601–604, 1991.
11. Dizdaroglu M, Rao G, Halliwell B and Gajewski E, Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. *Arch Biochem Biophys* **285**: 317–324, 1991.
12. Stoewe R and Prutz WA, Copper-catalysed DNA damage by ascorbate and hydrogen peroxide: kinetics and yield. *Free Rad Biol Med* **3**: 97–105, 1987.
13. Dizdaroglu M and Bertold G, Characterisation of free radical-induced base damage in DNA at biologically relevant levels. *Anal Biochem* **156**: 182–188, 1986.
14. Dizdaroglu M, Holwitt E, Hagan MP and Blakely WF, Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide. *Biochem J* **235**: 531–536, 1986.
15. Dizdaroglu M, Dirksen ME, Jiang H and Robbins JH, Ionizing-radiation-induced damage in the DNA of cultured human cells. *Biochem J* **241**: 929–932, 1987.
16. Dizdaroglu M, Chemical characterisation of ionizing radiation induced damage to DNA. *BioTechniques* **4**: 536–546, 1986.
17. Floyd RA, West MS, Eneff KL, Schneider JE, Wong PK, Tingey DT and Hogsett WE, Conditions influencing yield and analysis of 8-hydroxy-2-deoxyguanosine in oxidatively damage DNA. *Anal Biochem* **188**: 155–158, 1990.
18. Engle CG, Lasinski E and Gelzer J, Rifampin inhibition of H-1 virus infection in Hamsters. *Nature* **228**: 1190–1191, 1970.