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OXIDATIVE DAMAGE TO DNA AND DEOXYRIBOSE BY β-LACTAM ANTIBIOTICS IN THE PRESENCE OF IRON AND COPPER SALTS

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β-lactam antibiotics in the presence of certain metal ions damage deoxyribose and DNA with the release of thiobarbituric acid-reactive material. This damage can be substantially prevented by catalase, metal chelators and some scavengers of the hydroxyl radical. Ferric salts in the presence of certain β-lactam antibiotics were effective in degrading deoxyribose but they did not appear to damage DNA. In contrast copper salts and β-lactam antibiotics were extremely effective in damaging both DNA and deoxyribose.

KEY WORDS: β-lactam antibiotics, DNA, deoxyribose, oxidation.

INTRODUCTION

Modern antibiotic chemotherapy began sixty years ago with the chance observation that a contaminating penicillin mould could produce lytic effects on staphylococci (for a review see Selwyn, 1980).[1] The penicillin nucleus, 6-aminopenicillanic acid and the cephalosporin nucleus 7-aminocephalosporic acid were isolated by 1960 and many semi-synthetic β-lactam antibiotics were subsequently prepared for clinical use.

β-lactam antibiotics are said to act by inhibiting key enzymes in the bacterial cell thereby preventing transpeptidation between peptidoglycan chains. However, this is probably an oversimplification of their *in vivo* mode of action on bacteria. Two recent proposals help broaden our view of antibiotic action and might explain how many antibiotics can exert measurable effects on the growth of bacteria when present *in vivo* at concentrations well below their *in vitro* minimum inhibitory concentrations (MIC). One proposal suggests that "superior antibiotic activity" *in vivo* results from a 'host effect' in which the antibiotic acts to trigger a host response that ultimately eradicates the infection.[2] The second hypothesis proposes that chemotherapeutic agents mediate electron transfers and oxidative damage to 'foreign cells' *in vivo* by using mechanisms similar to those evolved in phagocytic cells for the same purpose namely, cell killing by using reduced oxygen intermediates.[3]

Here, we extend our studies on anthracycline,[4] aureolic acid,[5] amino quinone[6] and tetracycline antibiotics[7] to show that β-lactam antibiotics (Figure 1) have also the potential to mimic phagocytic cells by producing reactive oxygen species that might act as trigger molecules or cell-killing agents.

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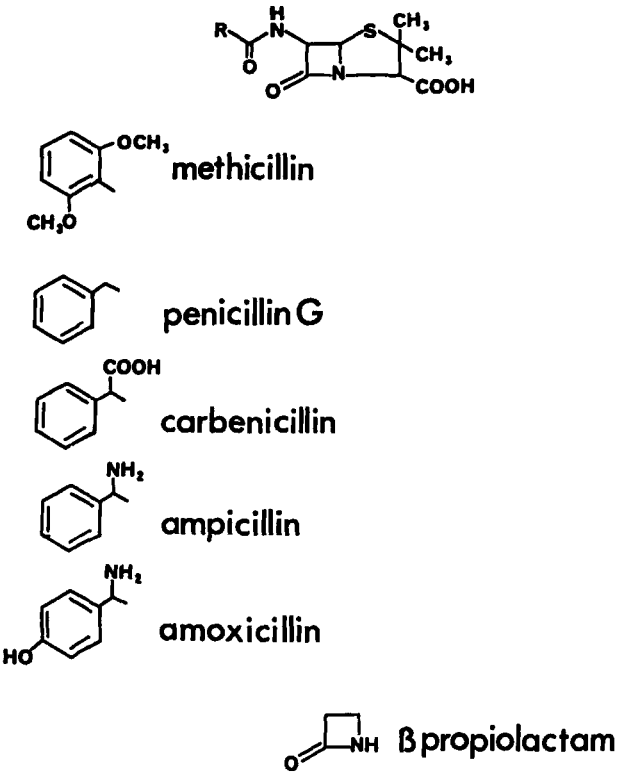

FIGURE 1 Structures of the β-lactam antibiotics.

MATERIALS AND METHODS

DNA (salmon testes), diethylenetriaminepentaacetic acid (DETAPAC), 2 deoxy-D-ribose, penicillin-G, methicillin, carbenicillin, ampicillin, amoxicillin, cephalosporin-C, cefazolin, cephradine, catalase (bovine liver), superoxide dismutase (bovine blood), albumin (human fatty acid free), penicillinase (Bacillus cereus), diethyldithiocarbamate were obtained from the Sigma Chemical Company, Poole, Dorset, 2-Azetidinone (β-propiolactam) from the Aldrich Chemical Company, Poole, Doreset. Desferrioxamine mesylate, Ciba-Geigy. All other chemicals were of the highest purity available from BDH Chemicals, Poole, Doreset.

Degradation of Deoxyribose and DNA

To new clean glass tubes were added the following reagents in the sequence indicated. 0.5 ml 0.1 M phosphate buffer pH 7.4 in 0.15 M NaCl. 0.2 ml of DNA 1 mg/ml or deoxyribose 10 mM, 0.2 ml of 5 mM antibiotic as indicated. 0.1 ml of 1 mM Cu(II) chloride or Fe(III) chloride. Where indicated in appropriate Figures or Tables inhibitors or scavengers were added at concentrations shown (addition concentrations). Final volumes were corrected by reducing the volume of buffer added. Scavengers and

TABLE 1
The effect of inhibitors on deoxyribose degradation by methicillin and a ferric salt

	Deoxyribose Degradation	
	TBA-reactivity as units of fluorescence at 553 nm (RFI)	% Inhibition
(1) Test deoxyribose + 1 mM ferric chloride + methicillin	101	
(2) Control deoxyribose + methicillin	23	
(3) Test + Benzoate 200 mM	75	25.75%
(4) Test + Formate 200 mM	79	21.8%
(5) Test + Urea 20 mM	101	0%
(6) Test + Thiourea 20 mM	19	81.2%
(7) Test + Mannitol	3	97%
(8) Test + DEFOM 2 mM	00	100%
(9) Test + EDTA 2 mM	31	69.7%
(10) Test + DETAPAC 2 mM	7	93%
(11) Test + Catalase .5 mg/ml	00	100%
(12) Test + Catalase .5 mg/ml HD	93	8%
(13) Test + SOD .5 mg/ml	73	27.7%
(14) Test + SOD .5 mg/ml HD	91	10%
(15) Test + Albumin .5 mg/ml	89	11.9%
(16) Test + Albumin .5 mg/ml HD	93	8%
(17) Deoxyribose + Fe^{3+} alone	0	–
(18) Methicillin + Fe^{3+} alone	0	–

Addition concentrations are shown and the results expressed as relative fluorescence intensity (RFI) units against a standard of rhodamine B. DEFOM – desferrioxamine; EDTA – ethylenediamine-tetraacetic acid; DETAPAC – diethylenetriaminepentaacetic acid; SOD – superoxide dismutase; HD – heat denatured. Values shown are corrected for the control value.

inhibitors were added before the metal salts. Reaction mixtures were incubated for three hours at 37°C in a shaking water bath.

Development of Thiobarbituric Acid-reactivity (TBA)

After incubation, 0.5 ml of TBA (1% w/v) in 0.05 M NaOH and 0.5 ml of 2.8% (w/v) trichloroacetic acid (TCA) were added to reaction mixtures containing deoxyribose. To samples containing DNA 25% (v/v) hydrochloric acid was added instead of TCA. All reaction mixtures were heated at 100°C for 15 mins. When cool, the resulting pink chromogen was extracted into 1 ml of butan-l-ol. Fluorescence was measured, using a spectrofluorimeter, at 553 nm with excitation at 532 nm. Fluorescence was expressed as units of 'relative fluorescence intensity' (RFI) calibrated with a standard of rhodamine B (3 μM).

Measurements with the Oxygen Electrode

A Clarke-type electrode (Hanstech Ltd) was calibrated with 100% air saturation at 35°C equal to 0.219 μ moles of oxygen/ml. In the electrode reaction vessel was placed 1.6 ml of phosphate-saline buffer pH 7.4, 0.4 ml of antibiotic 5 mM and 0.2 ml of Copper(II) or Iron(III) chloride 1 mm. The electrode was allowed to stabilize before the addition of the metal salt. Oxygen uptake was monitored for 40 mins.

TABLE 2
Deoxyribose and DNA degradation by a copper salt and β-lactam antibiotics as fluorescent TBA units

	TBA-reactivity as RFI units					
	Penicillin G		Methicillin		Carbenicillin	
	DR	DNA	DR	DNA	DR	DNA
Antibiotic + deoxyribose or DNA + Copper salt	49.5	22.5	55.5	21.5	28.0	10.5
Antibiotic + deoxyribose or DNA	4.0	2.0	10.5	2.5	5.5	2.0
Deoxyribose or DNA	5.0	3.5	1.0	1.0	1.0	1.0
Antibiotic + copper salt	2.5	15.0	2.0	1.0	2.5	1.0

RFI = relative fluorescence units; DR = deoxyribose. Concentrations of reagents are given in the methods section as addition concentrations and the results shown are the mean of 3 or more different assays.

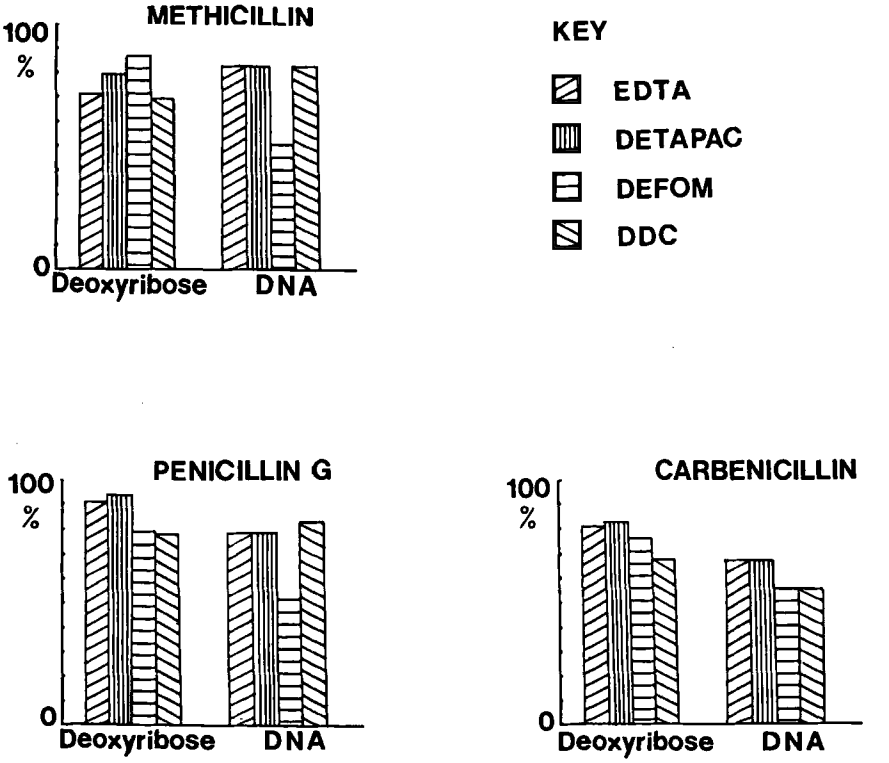

FIGURE 2 The percentage inhibition by metal chelators of deoxyribose and DNA degradation in the presence of β-lactam antibiotics and a copper salt. The fluorescence units upon which percentage inhibition was calculated are shown in Table 2.

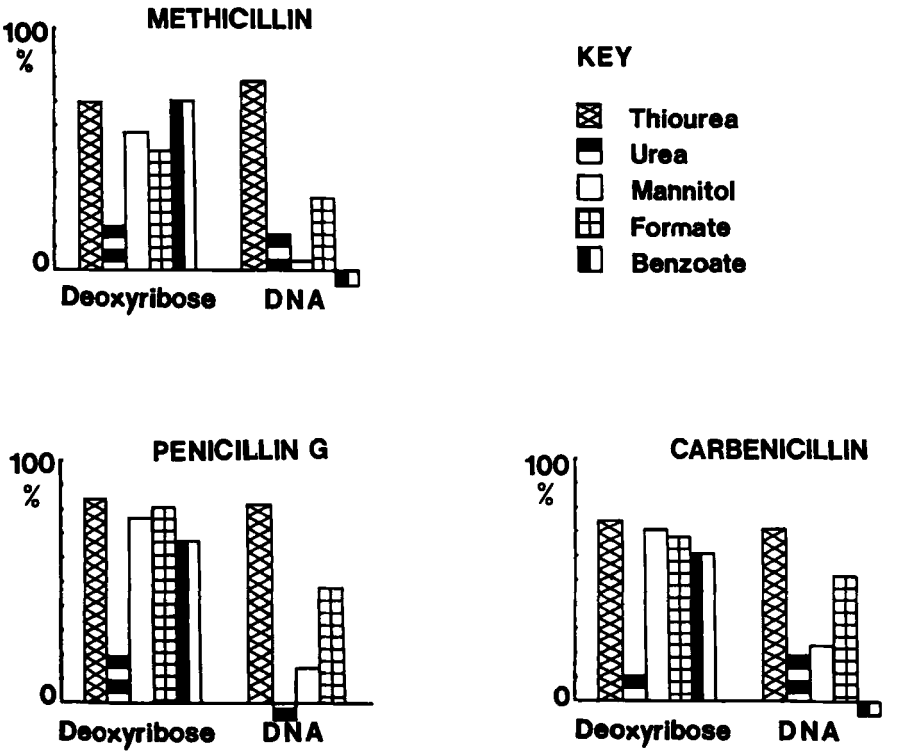

FIGURE 3 As for Figure 2 but showing percentage inhibition by various scavengers.

Electrophoresis of DNA

A flat bed agarose gel (0.45% w/v) was prepared in 0.1 M phosphate buffer pH 7.8 containing 5 mM EDTA. Samles were treated as described for degradation studies and diluted with an equal volume of molten agarose for loading into 25 μl wells in the gel. The gel was electrophoresed for 18 hrs at 18 mA. Resulting bands were developed in ethidium bromide (5 μg/ml) and viewed under UV lighting.

All results shown are the mean of three or more assays which differed by less than $\pm$ 5%. Concentrations are shown as the addition concentration of reactants.

RESULTS

The β-lactam antibiotics penicillin G, methicillin and carbenicillin, but not cephalosporin C, cephradine, cefazolin, amoxycillin or ampicillin, were able in the presence of a ferric salt to release thiobarbituric acid-reactive material (TBA) from the carbohydrate deoxyribose. Damage to deoxyribose could be substantially prevented by addition of catalase, iron chelators and certain scavengers of the hydroxyl radical such as thiourea and mannitol (Table 1) suggesting a key involvement of hydrogen peroxide and iron ions in the formation of a reactive intermediate with properties similar to the hydroxyl radical (·OH). A typical set of results are shown in Table 1

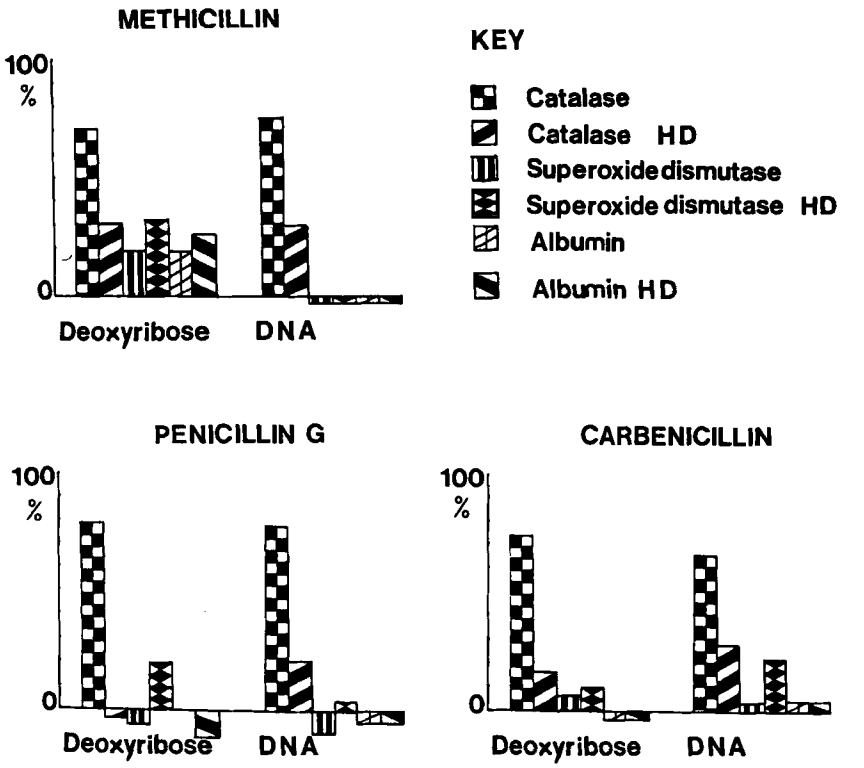

FIGURE 4 As for Figure 2 but showing percentage inhibition by proteins.

for the antibiotic methicillin although, similar patterns of inhibition were obtained for the other antibiotics listed above.

When DNA was substituted for deoxyribose in the above assay no TBA-reactivity was released from the sugar moiety of DNA and gel-electrophoresis showed that there was no change in the electrophoretic mobility of DNA after incubation with the β-lactam antibiotics and an iron salt.

If a copper salt was used instead of an iron salt then penicillin G, methicillin, carbenicillin, cephalosporin C, cephradine and cefazolin, but not amoxycillin or ampicillin, were able to degrade the sugar deoxyribose and the DNA molecule with release of TBA-reactive material (Table 2). Substantial damage to the DNA molecule had also occurred when it was monitored by changes in its electrophoretic mobility (Figure 5). When the copper salt concentration was increased 12-fold however, some TBA-reactive material was released from deoxyribose and DNA by amoxycillin and ampicillin (data not shown). Damage to deoxyribose and DNA was substantially inhibited by catalase (Figure 4), metal chelators (Figure 2) and certain scavengers of the ·OH radical (Figure 3) but not greatly by superoxide dismutase or albumin which was added as a protein control for non-specific scavenging effects (Figure 4). The inhibitory activity of catalase could be abolished by heat-denaturation of the protein.

With oxygen-uptake measurements we observed no increase in oxygen consumption when an iron salt was present with a β-lactam antibiotic. However, copper salts

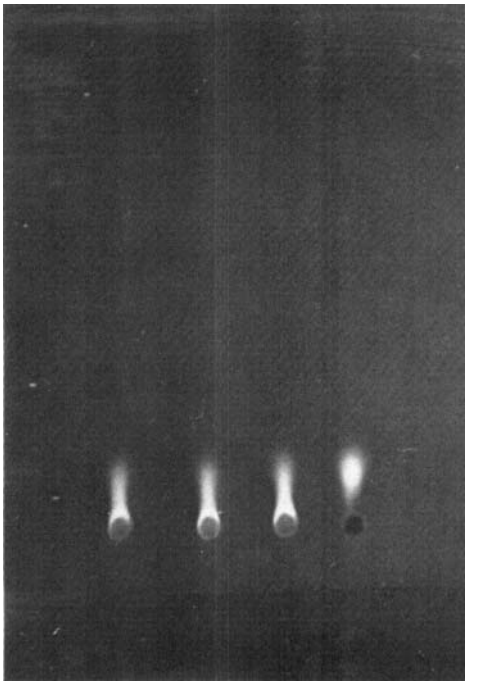

FIGURE 5 Electrophoretic separation of DNA degraded by a copper salt plus methicillin. 1. = DNA only; 2. = DNA + copper salt (0.1 mM final concentration); 3. = DNA + methicillin (1 mM final concentration); 4. = DNA + methicillin + copper salt.

greatly stimulated oxygen uptake and the pattern for the different antibiotics closely paralleled activity (Figure 6) seen with deoxyribose and DNA degradation. The synthetic mono-cyclic β-lactam β-propiolactam (Figure 1) was unable to produce damaging species in the presence of either iron or copper salts (data not shown).

Careful inclusion of assay controls showed that the antibiotics cephalosporin C and cefazolin were themselves able to generate TBA-reactive material from their molecules in the absence of deoxyribose or DNA. Amoxycillin and ampicillin showed a similar property when incubated with high concentrations of copper salt (12 mM), suggesting formation of aldehydic fragments from the antibiotics.

Incubation of β-lactam antibiotics with the enzyme β-lactamase abolished their ability to produce TBA-reactive material from deoxyribose in the presence of a copper salt. However, a heat-denatured β-lactamase control behaved similarly. Chemical degradation of penicillin G to penicillinoic acid[15] did not prevent the subsequent degradation of deoxyribose in the presence of a copper salt, however, suggesting that the β-lactam bond is not necessary for the damaging reactivity described here.

DISCUSSION

The β-lactam antibiotics examined here, with the exception of ampicillin and amoxycillin, were able to produce reactive oxygen intermediates in the presence of low concentrations of copper salts. Ampicillin and amoxycillin are distinct in containing

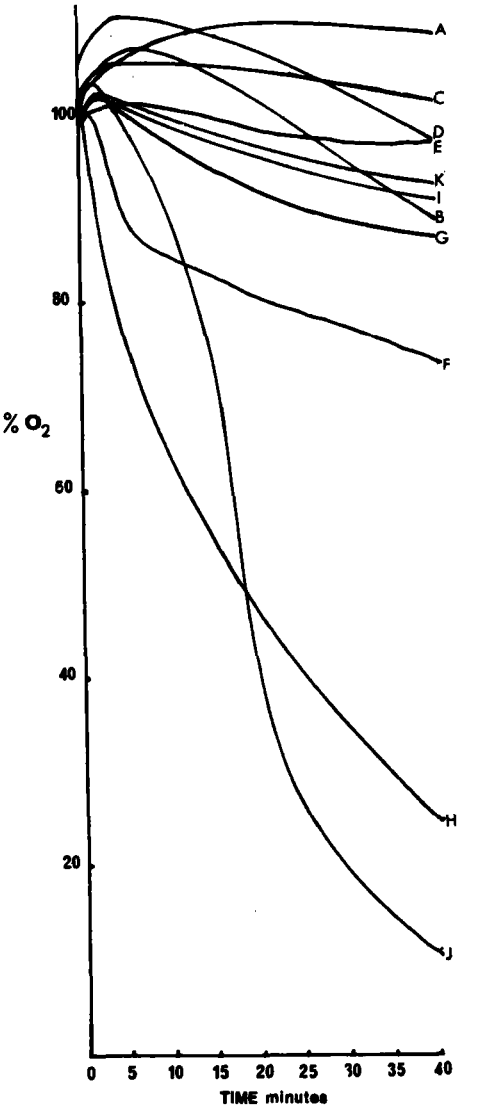

FIGURE 6 Oxygen uptake (%) by β-lactam antibiotics at 37°C. A = amoxicillin only (0.9 mM final concentration); B = amoxicillin + copper salt (0.09 mM final concentration); C = ampicillin only (0.9 mM); D = ampicillin + copper salt (0.09 mM); E = carbenicillin only (0.9 mM); F = carbenicillin + copper salt (0.09 mM); G = penicillin G only (0.9 mM); H = penicillin G + copper salt (0.09 mM); I = methicillin only (0.9 mM); J = methicillin + copper salt (0.09 mM).

a primary amino group in their structure and, it is likely that this group would be particularly effective at binding copper ions. Copper-binding could prevent copper ions from redox cycling or alternatively it may site-direct copper-dependent damage away from the detector molecules DNA and deoxyribose. A similar explanation may also apply to iron ions.

In all systems studied, the formation from the antibiotics of hydrogen peroxide was a key intermediate necessary for damage to DNA or deoxyribose to occur. This finding is in agreement with the behaviour of other antibacterial agents such as the rifamycins,[8] tetracyclines,[7] as well as most of the antitumour antibiotics.[4–6] However, only the β-lactams and rifamycins release TBA-reactive material from DNA in the presence of copper ions,[8] an unusual property shared with the antitumour antibiotic bleomycin in the presence of iron ions.[9] Unlike reactions involving antibiotics containing a quinone-group,[5] superoxide does not appear to be involved in the reduction of metal ions necessary for Fenton chemistry to occur. It has recently been proposed that the β-lactam antibiotics owe their redox reactivity to electron transfer reactions which involve the cationic iminium ion.[10] The mechanisms involved here are not yet established but can be summarized by the overall reaction:

$$\beta\text{-lactam (reduced)} + Fe^{3+} \text{ (or } Cu^{2+}) + O_2 + 2H^+ \quad (1)$$
$$\rightarrow \quad \beta\text{-lactam (oxidised)} + Fe^{2+} \text{ (or } Cu^{+}) + H_2O_2$$

$$Fe^{2+} \text{ (or } Cu^{+}) + H_2O_2 \rightarrow Fe^{3+} \text{ (or } Cu^{2+}) + OH^- + \cdot OH \quad (2)$$

The reasons why some scavengers of ·OH are effective and others are not probably relates to their metal-bindings properties and have been discussed in detail elsewhere.[11,12] Studies with the model β-lactam unit β-propiolactam and with the β-lactam cleaving enzyme β-lactamase showed that the β-lactam group alone was not essential for chemical activation of the antibiotics.

There is now increasing evidence that many antibiotics do not act solely as pharmacological agents blocking key enzymes and metabolic functions of bacteria but, that they also possess the ability to redox cycle and produce reactive intermediates capable of damaging the molecular structure of cells. Several products of redox cycling could be implicated in cell damage or in host mediated responses. These include superoxide and hydrogen peroxide, and their participation in the formation of more reactive species, as well as aldehydic fragments from the antibiotic.

In support of the suggestion that antibiotics might manipulate the body's defences by producing reactive intermediates we can cite the observation that it is often the body's reaction to infection rather than the infection itself that causes much of the tissue damage observed.[2] For example, it is possible that immune suppression is an unwelcome consequence of host response and is not directly caused by the infective agent itself. Although complexable iron and copper ions are not normally present in biological systems,[13] they can readily become released from safely sequestered sites when drug redox cycling generates excessive amounts of reduced oxygen intermediates.[14] Thus, it is possible for redox cycling drugs to have a 'phago-mimetic' action on bacterial cells.

References

1. Selwyn, S. The Beta-lactam Antibiotics. Hodder and Stroughton: London (1980).
2. O'Grady F. 'Antibiotics and Host-defences'. In The Scientific Basis of Antimicrobial Chemotherapy. The Society for General Microbiology, Symposium 38. Cambridge University Press: Cambridge. (1985) pp. 341–366.
3. Ames, J.R., Hollstein, U., Gagneux, A.R., Ryan, M.D. and Kovacic, P. *Free Rad. Biol. Med.*, **3,** 85–96, (1987).
4. Gutteridge, J.M.C. and Toeg, D. *FEBS Lett.* **149,** 228–232, (1982).
5. Gutteridge, J.M.C. and Quinlan, G.J. *Biochem, Pharmacol.* **34,** 4099–4103, (1985).

6. Gutteridge, J.M.C. *Biochem. Pharmacol.* **33,** 3059–3062, (1984).
7. Quinlan, J.G. and Gutteridge, J.M.C. *Free Rad. Biol. Med.* In the press (1988).
8. Quinlan, J.G. and Gutteridge, J.M.C. *Biochem. Pharmacol.* **36,** 3629–3633, (1987).
9. Sansville, F.A., Peisach, J. and Horwitz, S.B. *Biochem. Biophys. Res. Commun.* **73,** 814–822, (1976).
10. Kovacic, P., Jawdosink, M., Ames, J.R. and Ryan, M.D. *Bioorganic Chem.* **15,** 423–441, (1987).
11. Gutteridge, J.M.C. *Biochem. J.* **224,** 761–767, (1984).
12. Gutteridge, J.M.C. *Biochem. J.* **243,** 709–714, (1987).
13. Gutteridge, J.M.C. and Halliwell, B. *Life Chem. Reports,* **4,** 113–142, (1987).
14. Gutteridge, J.M.C. *FEBS Lett.,* **201,** 291–295, (1986).
15. Thomas, A.H. and Broadbridge, R.A. *Analyst,* **95,** 459–462, (1970).

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