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SHORT COMMUNICATIONS

Hydroxyl-free radicals and anti-inflammatory drugs: biological inactivation studies and reaction rate constants

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Oxygen-free radicals are being increasingly implicated in the development of inflammatory disease [1–6]. The hydroxyl radical ($OH^{\cdot}$) in particular has been suggested as responsible for the degradation of synovial fluid occurring in rheumatoid arthritis. Recently it has been suggested that those anti-inflammatory compounds such as α-tocopherol (vitamin E) and the phenolic derivative MK-447, which have not been shown to inhibit prostaglandin biosynthesis in animal bodies, may exert their action by direct free radical scavenging.

The radiolysis of dilute aqueous solutions is becoming increasingly the method of choice of investigating reactions of hydroxyl radicals *in vitro*. When such solutions are saturated with nitrous oxide (N_2O) and exposed to γ-rays or high energy electrons, hydroxyl radicals constitute approximately 90% of the reactive species formed. For a radiation dose of 100 rad (1 Gy or 1 J/kg) the yield of hydroxyl radicals is approximately 0.3 μM.

We now report studies in which bacteriophage viability has been used as an end-point to assess the protective ability of non-steroidal anti-inflammatory drugs (NSAIDs) against biological damage induced by hydroxyl radicals. The high rate constant for the reaction of the compounds with the oxidizing free radical has been confirmed using the fast reaction technique of pulse radiolysis.

Materials and methods

Aspirin (acetyl salicylic acid, Sigma), D-penicillamine (Sigma), indomethacin (Sigma), flurbiprofen (Boots) and metiazinic acid (May & Baker) were used without further purification (Fig. 1). Bacteriophage T_2 was obtained as broth lysate from The National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. (NCIB 10358) and grown in minimal nutrient broth medium according to standard procedures [7]. After an additional double washing with phosphate buffer the phage were suspended in 1/15 M phosphate buffer (pH 7) at a concentration of 3.2×10^{10} plaque-forming particles (p.f.p) per ml and kept at 4°. For the inactivation experiments the stock solution was diluted to approximately 10^7 p.f.p./ml. The infective ability of the phage was assayed according to the standard double layer method [8]. All assays were carried out in triplicate with typical values for S.D. from 5 to 10%.

Phage suspensions were irradiated using the Brunel 2000 Ci ^{60}Co source at a distance of 16 cm. The dose rate determined using the Fricke dosimeter [9] was approximately 6.7 Gy/min.

Pulse radiolysis experiments were undertaken using the Brunel 4 MeV linear accelerator delivering pulses of 200 nsec duration and generating hydroxyl radicals from nitrous oxide-saturated water in a concentration range 1–20 μM [10]. All experiments were carried out at room temperature and in 1/15 M phosphate buffer (pH 7) made up with 'Millipore'-filtered water.

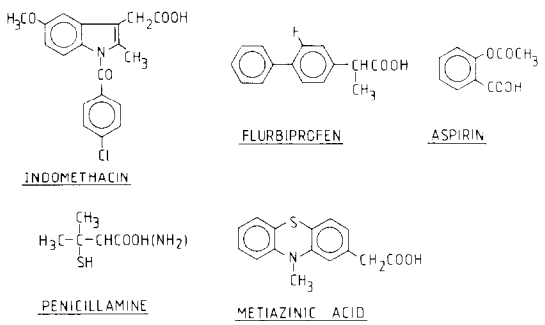

Fig. 1. Structural formula of the NSAID.

Results

Phage inactivation studies. The radiation-induced inactivation of bacteriophage T_2 under conditions where the hydroxyl radical is the principal reactive species is shown in Fig. 2. After a radiation dose of 17 Gy corresponding to a total $OH^{\cdot}$ yield of 10 nM/ml only 10% of the initially viable particles (10^7 p.f.p./ml) survive. Although the semi-logarithmic inactivation curve has a very small shoulder at low radiation dose, it is essentially linear over the range 10–50 Gy.

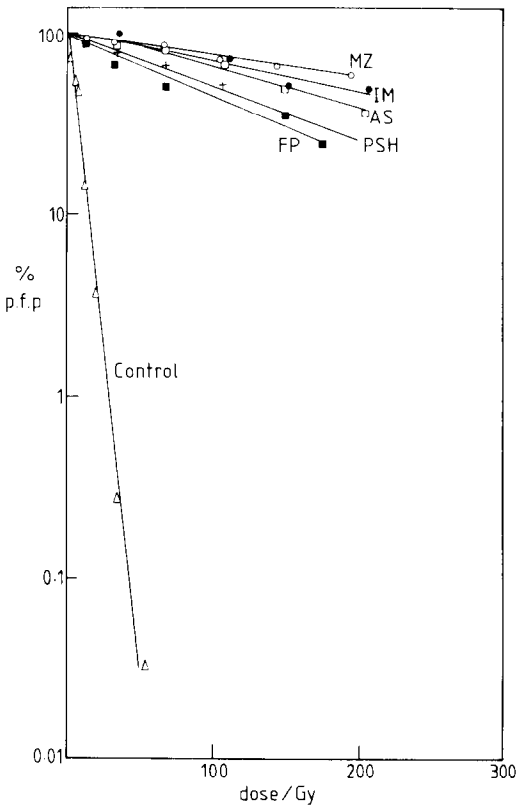

Fig. 2. Inactivation of bacteriophage T_2 by hydroxyl radicals in nitrous oxide-saturated phosphate buffer suspension, and protection by anti-inflammatory drugs (50 μM): metiazinic acid (MZ), indomethacin (IM), aspirin (AS), D-penicillamine (PSH), flurbiprofen (FP). Dose rate: 6.7 Gy/min.

Similar studies were undertaken in the presence of the NSAIDs metiazinic acid, indomethacin, flurbiprofen, D-penicillamine and aspirin. With 50 μM of the respective drugs, the extent of inactivation was considerably reduced, more than 20% of the phage surviving the maximum radiation doses used (200 Gy, total $OH^{\cdot}$ yield approximately 120 nM/ml).

Determination of hydroxyl radical rate constants. The absolute rate constant for the reaction of the hydroxyl radical with aspirin, indomethacin and flurbiprofen has been determined using the competition technique previously described [11]. On pulse radiolysis of N_2O-saturated solutions containing the peroxidase reagent 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonate), ABTS, 2×10^{-4} M, the characteristic radical cation absorption at 415 nm was observed and found to be progressively reduced in the presence of increasing concentrations of the drugs. The kinetic competition plots of the ratio of the absorption observed in the absence of the drugs (A°) to that observed in their presence (A) against the relative drug to ABTS concentration are shown in Fig. 3.

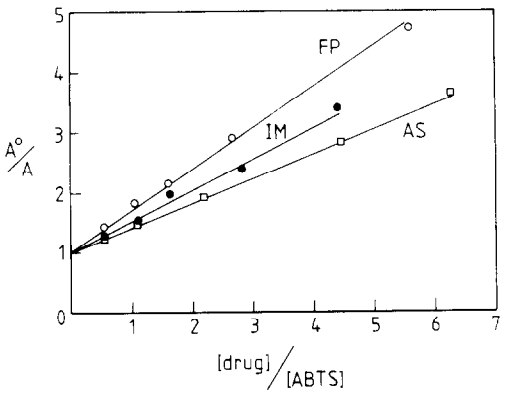

Fig. 3. Kinetic plots for competition for $OH^{\cdot}$ between 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonate), ABTS, and (a) flurbiprofen, (b) indomethacin and (c) aspirin, as reflected in the magnitude of the ABTS radical–cation absorption at 415 nm (see text).

The results are in agreement with the competing reactions

(1) $OH^{\cdot}$ + Drug → Products
No absorption at 415 nm

(2) $OH^{\cdot}$ + ABTS → $ABTS^{-\cdot}$ + products
λ_{max} = 415 nm

and the equation $A^{\circ}/A = 1 + k_1[\text{drug}]/k_2[\text{ABTS}]$. From the slopes of the plots for aspirin $k_1/k_2 = 0.42$, indomethacin $k_1/k_2 = 0.52$ and for flurbiprofen $k_1/k_2 = 0.65$ are calculated by the least-square fit treatment. Since the absolute rate constant $k_2 = 1.2 \times 10^{10}\ M^{-1}s^{-1}$ has been determined previously [11], absolute values for for the rates of reactions of the drugs can be calculated. These are shown together with the literature values for D-penicillamine and metiazinic acid (Table 1).

Table 1. Absolute rate constant data for the reaction of hydroxyl radicals with anti-inflammatory drugs

Drug	$k \times 10^{-9}$ ($M^{-1}s^{-1}$)
Aspirin	5.0
Indomethacin	6.2
Flurbiprofen	7.8
Metiazinic acid	8.4*
D-Penicillamine	5.0†

* From [12].
† From [13].

Discussion

The bacteriophage results described are clearly in agreement with the NSAIDs studied being efficient scavengers of hydroxyl radicals. The pulse radiolysis studies confirm that they do indeed react very rapidly with the radical at near diffusion controlled rates.

Whether this high reactivity towards $OH^{\cdot}$ has a bearing on the anti-inflammatory properties of these drugs remains a matter for debate; other biological compounds also react rapidly with this highly electrophilic species. It has been suggested, however, that the hydroxyl radical is generated during the enzymatic conversion of PGG_2 into PGH_2, the precursor of more stable prostaglandins. If these anti-inflammatory drugs are favoured over other compounds in being able to reach or to be selectively concentrated in the critical sites of the enzyme complexes involved, their higher $OH^{\cdot}$ reactivity could be particularly valuable.

The present phage studies also show that if the free radicals derived from the NSAIDs following their scavenging of $OH^{\cdot}$ react with the viral particles, then their damaging action is comparatively very small. This observation is particularly important in the context of free radical protection, as there is increasing evidence that secondary free radicals formed from a variety of biochemicals may themselves be damaging, indeed perhaps more so than the original radical of interest. The suggestion that some free radical scavengers such as cysteamine, methionine, cysteine and acetylcysteine should be considered as candidates for clinical trial against rheumatoid arthritis [15] is particularly pertinent in this respect. Recent pulse radiolysis studies indicate that secondary radicals derived from the reaction of $OH^{\cdot}$ with these compounds may themselves react with cellular constituents [11, 14]. Clearly, a full appraisal of the reactions of not only the primary but also the secondary, even subsequent free radicals, involved, is necessary before any authorative predictions can be made concerning clinical efficacy from *in vitro* studies.

In summary, inactivation of bacteriophage T_2 by hydroxyl-free radicals is strongly inhibited by the presence of the non-steroidal anti-inflammatory drugs (NSAIDs) metiazinic acid, indomethacin, flurbiprofen, D-penicillamine and aspirin. Pulse radiolysis studies have confirmed that the hydroxyl-free radical reacts rapidly with these drugs.

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Correlation between the affinity for [^{3}H]mianserin-labelled receptors in brain and antagonism of the serotonin pressor response in pithed rats

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Two distinct types of serotonin receptor binding sites have been identified in *in vitro* binding studies [1–3]. One class, the S_1-receptor, is specifically labelled by tritiated serotonin. Frontal cortex tissue contains the so-called S_2-receptor [2, 3], which was originally defined by the high affinity binding of [^{3}H]spiperone. At present two other ligands, [^{3}H]mianserin and [^{3}H]ketanserin, are available to investigate the affinity for the S_2-receptor. They are advocated to be more suitable for use as the S_2-ligand than [^{3}H]spiperone [4, 5].

The subdivision of serotonin receptors is of physiological relevance since the S_2-receptor, in contrast to the S_1-receptor, is associated with serotonin-induced blood vessel contraction [6], as assayed *in vitro*. Affinity for brian S_2-receptors, identified by [^{3}H]ketanserin, has been found to correspond with the antagonistic activity of numerous compounds towards serotonin-evoked vasoconstriction of the rat caudal artery [5]. A study of serotonin-mediated vasoconstriction *in vitro* has the advantage that the results are not affected by pharmacokinetic factors. This approach, however, has one major drawback—that the vessel which can be used in such an assay does not determine the peripheral resistance in an *in vivo* situation.

The objective of the present study was to investigate whether the affinity for brain S_2-receptors could be correlated with the inhibitory activity of antagonists towards the vasopressor effect of serotonin in the intact circulatory system of pithed rat. Affinity for brain S_2-receptors was determined by the method of Peroutka and Snyder [4] using [^{3}H]mianserin.

Materials and methods

Animals. Male Wistar normotensive rats (200–250 g) were used throughout the study. The animals had been kept at a standardized diet of normal chow (Muracon), and water *ad lib.*

Radioligand binding assay. Selective labelling of S_2-receptors was performed using the method of Peroutka and Snyder [4]. In brief, after decapitation, brains were quickly removed and the frontal cortex was dissected on ice. This tissue was homogenized in ice-cold Tris–HCl buffer (pH 7.7). The homogenate was centrifuged at 50,000 *g* for 10 min, and washed twice with buffer. If necessary the pellet was stored at −20°. Incubation tubes contained 0.2 ml of 10^{-9} M [^{3}H]mianserin, 0.5 ml tissue suspension containing 2 mg protein/ml, 0.1 ml of 3×10^{-6} M triprolidine and a 0.2 ml solution of the drug to be tested. All assays were performed in triplicate. After incubation at 25° for 45 min, labelled membranes were collected by rapid vacuum filtration through Whatman GF/B filters and washed with three 4 ml rinses of ice-cold Tris–HCl buffer. The filters were transferred to glass counting bottles and left to solubilize in 10 ml Hydrocount® (Baker Chemicals, Philipsberg, PA) for 24 hr at room temperature and finally counted at an efficiency of about 40%. Specific binding of [^{3}H]mianserin was defined as the excess over blanks containing 1 μM cyproheptadine. Specific binding accounts for approximately 60% of total binding at a concentration of 0.2 nM, the amount of [^{3}H]mianserin used in routine drug competition studies.

Antagonism towards serotonin in vivo. When administered i.v. to pithed normotensive male Wistar rats, serotonin evokes a dose-dependent pressor response [7]. This increase in blood pressure is selectively inhibited by serotonin antagonists [7, 8]. ED_{50} values for antagonistic potency of drugs were determined as follows. Pithed rats received an i.v. control dose of 100 μg/kg serotonin as a bolus injection. Pre-treatment (i.v.) with a low dose of antagonist was started and 15 min later serotonin (100 μg/kg) was re-administered. As soon as the diastolic pressure passed its maximal value, another appropriate additive amount of antagonist was given, and 15 min later serotonin was injected again. This procedure was repeated once. Each animal therefore received three doses of antagonist in a cumulative order (e.g. 1 + 2 + 7 mg/kg). Control experiments had shown that in the course of 90 min the repeated administration of 100 μg/kg serotonin at 15 min intervals elicited identical pressor responses. The increases in diastolic pressure observed after the administration of antagonist were transformed into percentages of the control response. Regression analysis of these values against the respective log dose of the antagonist yielded the ED_{50} value, i.e. the dose (mole/kg) of antagonist causing a reduction of the initial pressor responses to 100 μg/kg serotonin by 50%. At least six different rats were used for one antagonist.

Drugs. [^{3}H]mianserin (55 Ci/mmole) was purchased from New England Nuclear (Boston, MA). The ligand was stored at −20° and diluted in Tris–HCL buffer to a concentration of 10^{-9} M immediately before use. Unlabelled drugs were