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Peroxidase-catalyzed Effects of Indole-3-Acetic Acid and Analogues on Lipid Membranes, DNA, and Mammalian Cells *In Vitro*

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ABSTRACT. This study aimed to explore the mechanisms and molecular parameters which control the cytotoxicity of derivatives of indole-3-acetic acid (IAA) when oxidatively activated by horseradish peroxidase (HRP). Lipid peroxidation was measured in liposomes, damage to supercoiled plasmid DNA assessed by gel electrophoresis, free radical intermediates detected by EPR following spin trapping, binding of IAA-derived products demonstrated by ^{3}H labelling, stable products measured by HPLC, and cytotoxicity in hamster fibroblasts measured by clonogenic survival. IAA, and nine analogues more easily oxidized by HRP, caused lipid peroxidation in liposomes, but not detectably in membranes of hamster fibroblasts, and were cytotoxic after HRP activation to varying degrees. Cytotoxicity was not correlated with activation rate. The hydrophilic vitamin E analogue, Trolox, inhibited cytotoxicity, whereas loading fibroblasts with vitamin E was ineffective, consistent with an oxidative mechanism in which radical precursors to damage are intercepted by Trolox in the aqueous phase. However, two known oxidation products were nontoxic (the 3-carbinol and 3-aldehyde, both probably produced from 3-$CH_2OO^{\cdot}$ peroxyl radicals via the 3-$CH_2^{\cdot}$ [skatolyl] radical following decarboxylation of the radical cation). The skatolyl radical from IAA was shown by EPR with spin trapping to react with DNA; electrophoresis showed binding to occur. Treatment of hamster fibroblasts with 5-^{3}H-IAA/HRP resulted in intracellular bound ^{3}H. Together with earlier results, the new data point to unknown electrophilic oxidation products, reactive towards intracellular targets, being involved in cytotoxicity of the IAA/HRP combination, rather than direct attack of free radicals, excited states, or membrane lipid peroxidation. BIOCHEM PHARMACOL **57**;4:375–382, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. indole acetic acid; horseradish peroxidase; skatolyl radical; lipid peroxidation; DNA binding

Haem peroxidases, such as that from horseradish (HRP†, donor:hydrogen peroxide oxidoreductase, E.C. 1.11.1.7), oxidize IAA, a reaction important in plant physiology [1–10]. Myeloperoxidase, abundantly produced by activated neutrophils and myeloid leukaemia cells, may also catalyze the oxidation of IAA [11, 12]. Radical fragmentation and involvement of skatolyl and peroxyl radicals on oxidation of IAA were discussed many years ago [2]. Later studies on this reaction [6, 13] and on IAA oxidation in enzyme-free systems [14] have shown that the substrate radical cation is an intermediate which undergoes scission (directly observable [14, 15]) of the exocyclic carbon–carbon bond to yield a carbon-centred radical, the skatolyl radical. In the presence of oxygen, the latter is rapidly converted to a peroxyl radical (Fig. 1).

The HRP/IAA combination can cause lipid peroxidation in model lipid vesicles [16–18], and effects on nucleic acids have also been reported [19, 20]. This combination might therefore enhance cellular oxidative stress [16] or be a possible basis for enzyme/prodrug application to tumour therapy, involving antibody-directed enzyme–prodrug therapy ('ADEPT' [21]). Cytotoxicity towards mammalian cells *in vitro* by IAA/HRP treatment, but not IAA or HRP alone, was found in preliminary experiments [22]. Other work has associated cytotoxicity of IAA towards neutrophils with peroxidase activity [23]. However, the pathways responsible for cytotoxicity and the relationships between chemical structure and biological activity in analogues of IAA are unknown. In the present study, we have sought to identify the main characteristics of the species involved in cytotoxicity and whether the possible site of action involved extracellular, membrane-associated, or intracellular events.

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† *Abbreviations:* IAA, indole-3-acetic acid; HRP, horseradish peroxidase; EMEM, Eagle's Minimum Essential Medium; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; TBARS, thiobarbituric acid reactive substances; 1-Me-IAA, 1-methyl-IAA; 2-Me-IAA, 2-methyl-IAA; 1,2 diMe-IAA, 1,2-dimethyl-IAA; 5-MeO-IAA, 5-methoxy-IAA; 2-Me-5-MeO-IAA, 2-methyl-5-MeO-IAA; 1,2-diMe-5-MeO-IAA, 1,2-dimethyl-5-MeO-IAA; 2-Me-5,6-diMe-IAA, 2-Me-5,6-dimethoxy-IAA; IPA, indole-2-propionic acid; 1-Me-IPA, 1-methyl-IPA; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; and Skatole, 3-methyl-1*H*-indole.

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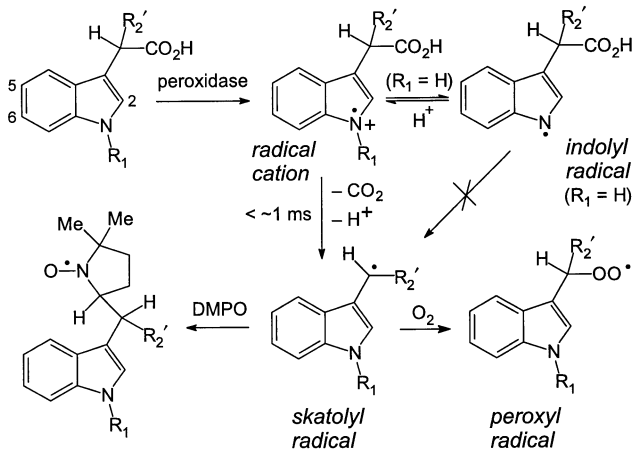

FIG. 1. Structures of IAA derivatives (when $R_2' = H$) showing the substituent positions and the reaction scheme for peroxidase-catalyzed decarboxylation via the radical cation, and reaction of the skatolyl radical with oxygen or the spin trap DMPO. (When $R_2' = CH_3$, compounds are derivatives of IPA).

MATERIALS AND METHODS

Materials

IAA, 2-Me-IAA, 2-Me-5-MeO-IAA, 5-MeO-IAA, indole-3-carbinol, indole-3-aldehyde, calf thymus DNA, and Trolox were obtained from Aldrich and used as received. Aqueous stocks of DMPO from Sigma were purified by charcoal filtration and stored at −20° under nitrogen [24]. 2-Me-5,6-DiMeO-IAA was prepared as described previously [9]. 1,2-diMe-IAA, 1,2-diMe-5-MeO-IAA, 1-Me-IPA, and 1,2-diMe-5,6-diMeO-IPA were obtained as previously described [25]. HRP type IV-A, α-tocopherol (T3251), phenol-red-free Hanks' balanced salt solution, and foetal bovine serum were obtained from Sigma. EMEM and Spinner-modified EMEM were from GIBCO. 5-^{3}H-IAA (aqueous solution, activity 37 MBq/mL) was obtained from Nycomed Amersham.

Methods

For cytotoxicity measurements, Chinese hamster lung fibroblast-like (V79-379A) cells were maintained in Spinner-modified EMEM in 7.5% foetal bovine serum in asynchronous logarithmic phase. Indole stock solutions (1 mM) were prepared daily in 10% v/v ethanol. Cells were treated for up to 2 hr on Petri dishes with 0.1 mM indole with or without 1.2 μg/mL HRP in phenol-red-free Hanks' balanced salt solution pH 7.4, under 5% CO_2/air at 37°. The cells were then washed with Hanks' and left to grow in EMEM with 10% serum for seven days. After this time, the cells were fixed with 75% methanol and stained with 1% w/v crystal violet; colonies containing >50 cells were counted. Surviving fraction was calculated relative to untreated controls. In some studies, the drug was incubated with the HRP in the absence of cells for 90 min. The resulting solution was ultrafiltered (Whatman Vecta, 20 kDa cut-off) to remove the enzyme, the filtrate applied to the cells for 90 min, and the toxicity assessed as above.

Products from treating indoles with HRP were analyzed by HPLC using a Hypersil 5ODS 125 × 4.6 mm column. Elution was achieved with a linear gradient of 27–100% in 5 min of 75% acetonitrile/20 mM potassium acetate, pH 4.5. Detection was by absorbance at 280 nm using a Waters 486 Variable Wavelength Detector.

Four methods were used to detect the effects of lipid peroxidation in cells. (*i*) The thiobarbituric acid test [26] was used to measure lipid hydroperoxides and malondialdehyde. 2×10^6 V79 cells/mL were incubated with stirring with 0.2 mM IAA or 2-Me-IAA and 10 μg/mL HRP in Hanks' solution for up to 40 min at room temperature. Oxidation was stopped by the addition of butylated hydroxytoluene (0.5 mg/mL). Parallel measurements using liposomes [17] confirmed the detection procedure. (*ii*) Malondialdehyde formation was measured by HPLC both before and after alkaline lysis of the cells or liposomes [27]. (*iii*) Lipid hydroperoxides in treated cells were also tested by the formation of an Fe(III)–thiocyanate complex with absorbance at 492 nm [28]. (*iv*) Loss of double bonds from unsaturated fatty acids and the loss of polyunsaturated fatty acids was measured by HPLC. 6×10^6 V79 cells/mL were treated with 0.2 mM IAA or 2-Me-IAA and 10 μg/mL HRP in PBS/glucose (1% w/v) for 1 hr. The cells were washed and internal standard (undecanoic acid) added. The lipids were extracted with chloroform/methanol and saponified with 2 mL NaOH (0.5 M) at 100° for 30 min. The soap was then acidified and extracted with hexane, the organic fraction washed with water and dried under nitrogen, and the lipids derivatized in acetone with 4-bromomethyl-7-methoxycoumarin (5 mM) and potassium carbonate (10 mg/mL) at 90° for 1 hr. Derivatized fatty acids were then measured by HPLC and compared to standard lipid samples using a Hypersil 5ODS 250 × 4.6 mm column. Elution was achieved with A: 5% tetrahydrofuran, 60% acetonitrile, 10% methanol, and B: 10% tetrahydrofuran, 70% acetonitrile, 20% methanol, with a linear gradient of 0–45% B in 25 min and 45–65% B in 15 min. Fluorescence detection was used (Perkin-Elmer LS40 Fluorescence Detector) with excitation at 323 nm and emission at 394 nm.

To study the effects of α-tocopherol (see [29]), V79 cells were plated for 1 hr in EMEM, the medium removed and replaced with α-tocopherol (2 mL/10 μM, from 10 mM stock in ethanol diluted in EMEM) for 2 hr at 37°, the cells washed with 2 mL EMEM, and IAA (2 mL 0.1 mM in Hanks') added for up to 2 hr. The cells were washed with 2 mL Hanks' and left to grow in 4 mL EMEM for 7 days before staining and counting as described above.

Cellular uptake of compounds [30] was measured by stirring 7.2×10^5 V79 cells/mL with 0.5 mM indole at 37° under 5% CO_2/air for up to 2 hr. Aliquots of 1.4×10^7 cells were taken into preweighed tubes at regular intervals. The cells were spun down and a 50 μL sample of the supernatant taken. The tubes containing the remaining pellet were

TABLE 1. The compounds studied

No.	Abbreviation	R_1	R_2	R_5	R_6	R'_2
1	IAA	H	H	H	H	H
2	1-Me-IAA	CH_3	H	H	H	H
3	2-Me-IAA	H	CH_3	H	H	H
4	1,2-diMe-IAA	CH_3	CH_3	H	H	H
5	5-MeO-IAA	H	H	CH_3O	H	H
6	2-Me-5-MeO-IAA	H	CH_3	CH_3O	H	H
7	1,2-diMe-5-MeO-IAA	CH_3	CH_3	CH_3O	H	H
8	2-Me-5,6-diMeO-IAA	H	CH_3	CH_3O	CH_3O	H
9	1-Me-IPA	CH_3	H	H	H	CH_3
10	1,2-diMe-5,6-diMeO-IPA	CH_3	CH_3	CH_3O	CH_3O	CH_3

See Fig. 1 for structure of IAA and substituent positions.

washed and dried, taking care not to disturb the pellet, and the tubes re-weighed to determine the pellet mass. Internal standard (indole-3-butyric acid, 50 μL, 1 mM) was added to the supernatants and pellets along with methanol (1 mL) to lyse the cells. The samples were spun (1500 *g* for 10 min) and the supernatants dried under nitrogen. The dry samples were then resuspended in HPLC eluent for chromatography. The uptake of the indoles was calculated as described previously [30]. Uptake of α-tocopherol was also measured: V79 cells (1×10^6) were plated in 6-cm dishes for 1 hr and α-tocopherol (20 μM) added for up to 2 hr at 37°. At intervals, cells were washed (Hanks', 5 × 2 mL) and lysed with trichloroacetic acid (5% w/v, 1 mL) and scraped off. Samples (0.5 mL) were added to butylated hydroxytoluene (1 mg/mL, 50 μL), mixed, SDS (0.5 mL, 0.1 M) added, mixed for 10 sec and ethanol (1 mL) and methanol (1 mL) added and mixed for a further 30 sec. The α-tocopherol was extracted in hexane (2 × 5 mL), dried down under N_2, reconstituted in methanol (0.2 mL), and measured by HPLC with electrochemical detection (Coulochem, ESA) using a dual porous graphite electrode (first electrode at −0.7 V; second [monitoring] at + 0.4 V). An eluent of 97.5% methanol/50 mM $NaClO_4$ (1.4 mL/min) was used with a Novapak 150 × 4 mm column.

IAA binding to whole cells after HRP treatment was measured after plating V79 cells (2×10^6) on to 10-cm Petri dishes for 1 hr. 5-^{3}H-IAA (7.4 kBq) diluted in 0.1 mM IAA in Hanks' solution (2.5 mL) was incubated with cells (and HRP, 1.2 μg/mL as required) for 2 hr at 37° under 5% CO_2/air. Cells were washed (×5) with Hanks', scraped off, resuspended in 1 mL Hanks' and 5 mL scintillant added (PCS, Nycomed Amersham + 0.7% acetic acid) before scintillation counting (Beckman LS6500).

Peroxidase-induced binding of indoles to DNA was tested by a plasmid band shift assay. PBR322 plasmids were isolated from *Escherichia coli* strain HB101 using a Quiagen Kit (Mega plasmid). Plasmid DNA (1 mg/mL) was incubated with 2 mM indole with or without HRP (6.7 μg/mL) for 1 hr at 37°. The samples were then loaded onto 1.4% agarose gels for electrophoresis in TBE buffer (89.2 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8) for 16 hr at 16 V, using a Bio-Rad mini subDNA cell with a model 200/2.0 power supply. The DNA was stained with ethidium bromide (0.5 μg/mL) and photographed over a UV light.

Reaction of the skatolyl radical with calf thymus DNA was investigated by X-band EPR spectrometry using a Bruker EMX spectrometer equipped with a TM resonator. IAA (1 mM), H_2O_2 (1 mM), HRP (50 μg/mL) at pH 5 (8 mM potassium acetate buffer) and DMPO (10 mM) was added to various concentrations of DNA. The intensity of the low-field line of the EPR spectrum of the skatolyl–DMPO adduct was monitored for ca. 15 min and the maximum intensity (ca. 1.5 min after the beginning of the reaction) was measured. Instrument conditions were: frequency, 9.78 GHz; centre field, 346.2 mT; power, 50 mW; modulation, 100 kHz/0.4 mT; receiver gain, 2×10^5; sweep, 10 mT/671 sec; conversion, 655 msec; time constant, 41 msec.

RESULTS

Cytotoxicity of Indole/Peroxidase Combinations

The toxicity to V79 cells of IAA and derivatives was tested using a clonogenic assay. None of the compounds listed in Table 1 showed any measurable cytotoxicity after 2-hr exposure at a concentration of 0.1 mM. Peroxidase (HRP) treatment on its own (10 μg/mL, 2 hr) was also not toxic, with or without ethanol (1% v/v), which was included when indoles were added. However, in combination with peroxidase, four of the compounds (IAA, 1-Me-IAA, 2-Me-IAA, and 2-Me-5-MeO-IAA, see Table 1) were cytotoxic (Fig. 2). Survival curves for IAA and two analogues have been reported [22] and an example is included in Fig. 3. Cytotoxicity from HRP (10 μg/mL) and IAA (0.1 mM) was similar with or without the 1% v/v ethanol used in most experiments from the stock solutions of indoles made up in 10% v/v ethanol. The remaining six compounds showed no detectable cytotoxicity, even in the presence of peroxidase. Two of the 'negative' compounds are derivatives of IPA; these are analogues of IAA with a methyl substituent on the exocylic carbon ($R'_2 = CH_3$, see Fig. 1 and Table 1).

Two main products of the oxidation of IAA are indole-3-carbinol and indole-3-aldehyde [6, 14]. The cytotoxicity

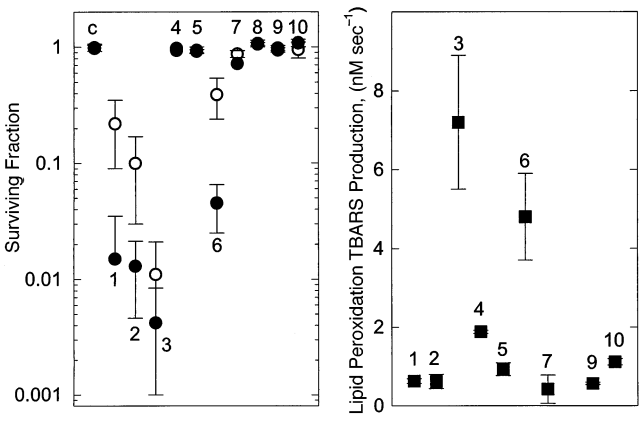

FIG. 2. Comparison of cytotoxicity (left) of the compounds when activated by peroxidase with lipid peroxidation in phosphatidylcholine liposomes induced by a similar treatment (right); c = controls (no compound, no enzyme), numbers refer to compounds in Table 1; surviving fractions are after 1.5 (○) or 2 hr (●); error bars are SD from means of 3 experiments (surviving fractions, each experiment is mean of 3 dishes).

of these compounds at a concentration of 0.05 mM (higher than that resulting from the complete oxidation of IAA in our experiments) was tested. No cytotoxicity was detected after 2 hr at 37° (data not shown).

Trolox, a water-soluble antioxidant with the same heterocyclic structure as α-tocopherol (vitamin E), completely eliminated the cytotoxicity of HRP with either IAA (Fig. 3) or 2-Me-IAA (data not shown) when present at a concentration of 10 μM. In contrast, loading the cells with α-tocopherol itself (2-hr incubation with 10 μM α-tocopherol, see below) had little effect on the cytotoxicity of HRP/IAA (Fig. 3) or alone (data not shown).

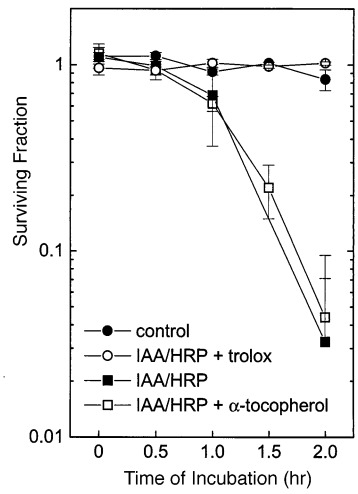

FIG. 3. Effect of inclusion of Trolox (10 μM) or of pretreatment with α-tocopherol (10 μM, 2 hr) on the cytotoxicity of IAA/HRP towards V79 cells. Data are means of 2 experiments each involving 3 samples (error bars are ± SD).

Uptake of a Representative Indole and of α-Tocopherol into V79 Cells

To check that the low cytotoxicity associated with 5-MeO-IAA/HRP treatment did not arise from poor intracellular uptake, the intracellular:extracellular concentration ratio (C_i/C_e) of this compound in V79 cells was measured. The compound rapidly achieved values consistent with equilibration across the cytoplasmic membrane and similar to those obtained with small, uncharged molecules of moderate lipophilicity [30]. Thus, values of $C_i/C_e \sim 0.8 \pm 0.1$ were obtained with 5-MeO-IAA after 30- to 120-min incubation.

α-Tocopherol was taken up by V79 cells with a loading of 8 ± 2 (SD) amol/cell after 2-hr treatment under the conditions used. This is consistent with an earlier report using this cell line, representing a doubling of the endogenous levels [29].

Lipid Peroxidation Induced by Indole/Peroxidase Combinations

Previously, we have shown that IAA and some derivatives together with peroxidase induce peroxidation of phosphatidylcholine liposomes, with formation of TBARS [16–18]. These experiments were extended to the other compounds listed in Table 1. The data are summarized in Fig. 2 to enable comparison of the differing abilities to induce lipid peroxidation with the effects on cell survival.

Several methods were used in an attempt to detect lipid peroxidation in V79 cells *in vitro*. However, no formation of lipid hydroperoxides or malonaldehyde, and no loss of cellular unsaturated fatty acids, could be detected with any of the compounds in the presence of HRP under conditions used to demonstrate cytotoxicity.

Peroxidase-catalyzed Binding of IAA to Mammalian Cells

Whole cells (V79 hamster fibroblasts) treated with IAA labelled with ^3H in the 5-position showed a 14-fold (14 ± 2) increase in label when cells were incubated with IAA and peroxidase, compared to IAA alone. Thus, controls (IAA, no peroxidase) had counts of 149 ± 21 dpm, compared to 2120 ± 440 dpm for IAA/HRP-treated cells.

Formation of DNA Adducts

Plasmid (supercoiled) DNA was incubated with indole-3-acetic acids and peroxidase, and analyzed by gel electrophoresis (Fig. 4). With some indole/peroxidase combinations, single- and double-strand breaks occurred, as shown by an increase in circular and linear DNA. In addition, the band of the supercoiled DNA treated with the same indoles was shifted towards higher molecular weights. The compounds causing most DNA damage when activated by HRP

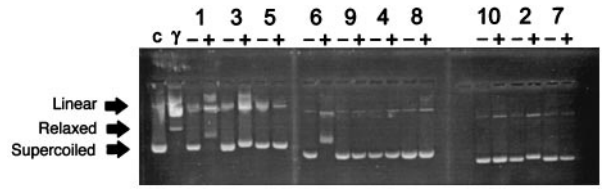

FIG. 4. Effect of indoles/peroxidase on supercoiled plasmid DNA, shown by gel electrophoresis. Lanes are: (c) control; (γ) control after 100 Gy γ-irradiation to show degradation behaviour; (−) DNA treated with compound alone; (+) DNA treated with compound + HRP; numbers above lanes refer to compounds in Table 1 (1 = IAA, 3 = 2-Me-IAA, 5 = 5-MeO-IAA, 6 = 2-Me-5-MeO-IAA, 9 = 1-Me-IPA, 4 = 1,2-diMe-IAA, 8 = 2-Me-5,6-diMeO-IAA, 10 = 1,2-diMe-5,6-diMeO-IPA, 2 = 1-Me-IAA, 7 = 1,2-diMe-5-MeO-IAA).

were IAA, 2-Me-IAA, 2-Me-5-MeO-IAA, and 1-Me-IAA (bands **1, 3, 6**, and **2**, respectively in Fig. 4).

Reaction of the Skatolyl Radical with DNA

IAA was incubated with HRP in the presence of DMPO; under these conditions, the skatolyl radical adduct to DMPO is detected [13]. In similar experiments, the corresponding EPR signal reached its maximum intensity ca. 1.5 min after the beginning of the reaction (Fig. 5, inset; in this lag phase, which includes the period before measurements began, oxygen is depleted in the sample through the formation of the skatole peroxyl radicals [13]). In the presence of DNA the signal intensity is decreased, suggesting that DNA quenches the skatolyl radical in competition with DMPO (Fig. 5). The maximum signal intensity measured in the presence of variable concentrations of DNA was used to derive an estimate of the ratio of the rates of reaction of the skatolyl radical with DNA (k_{DNA}) and with DMPO (k_{DMPO}), assuming simple kinetic competition: $k_{DNA}/k_{DMPO} = 36 \pm 6$. (The data in Fig. 5 are fitted to an exponential with equal weighting to all points, and hence the fitted curve does not pass through unity at zero time.)

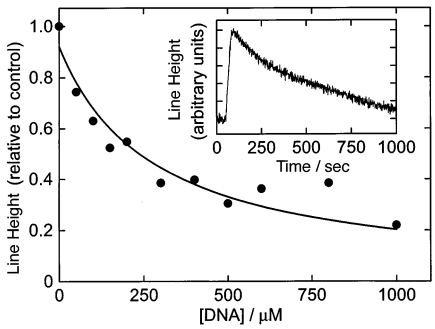

FIG. 5. Effect of double-stranded DNA on the maximum yield of the DMPO–skatolyl radical adduct measured on incubation of IAA in the presence of HRP/H_2O_2 and varying concentrations of DNA. The curve drawn is the fit assuming kinetic competition for the skatolyl radical reacting with either DMPO or DNA. Inset: time-course of radical signal.

DISCUSSION

IAAs are oxidized by HRP using peroxides (peroxidase activity) or oxygen (oxidase activity) as the oxidizing substrate [5, 6]. Since cytotoxicity towards hamster fibroblast-like cells was observed only in the presence of both indole and HRP, it is very likely to be associated with the oxidation of the indoles.

In our preliminary report of the cytotoxicity of the IAA/HRP combination towards mammalian cells [22], we discussed the importance of what we first assumed to be the most likely parameters which controlled the relative cytotoxicity of the different analogues of IAA in combination with HRP. Because there is a marked redox relationship in the activation of many substrates by peroxidases [31], the most likely candidate was the rate of prodrug oxidation by the oxidizing enzyme intermediates. This rate was modelled using the Compound I intermediate of HRP, which was shown to oxidize IAA and derivatives with rate constants defined by the reduction potential of the indole. Thus, HRP Compound I oxidized analogue **8** (2-Me-5,6-diMeO-IAA, Table 1) ~ 300-fold faster then IAA itself (**1**). However, **8** was not cytotoxic when activated by HRP (Fig. 2), whereas IAA (**1**) was. Examination of the additional data in Fig. 2 with the reported or likely oxidation rates by HRP (either reactivity of Compound I [9] or turnover of substrate [17]) fail to show any obvious correlation between HRP-activated cytotoxicity and the oxidation rates or redox properties. The main effects on radical reduction potential (or ease of oxidation of the indole) arise from ring substitution; methyl substitution on the exocyclic carbon (Fig. 1, $R_2' = CH_3$), as in compounds **9** and **10** (Table 1, IPA derivatives) would have a much smaller effect compared to substitution at positions 2, 5, and 6 on the indole ring (Fig. 1), and so **9** and **10** would be expected to be oxidized only slightly faster than IAA.

Since formation of skatole peroxyl radicals is probably implicated in the initiation of lipid peroxidation, as well as in other possible deleterious effects such as generation of singlet oxygen through the Russell mechanism [32] and the formation of the indole-3-aldehyde or other products in an excited state [11, 12, 19, 20, 33, 34], it is possible that fragmentation of the skatolyl radical cation (decarboxylation) is rate- (effect-) limiting. Decarboxylation of the radical cation is characterized by a dependence of rate constant on radical reduction potential, which is the inverse of that found for Compound I reactivity [25]; radical cations of analogues more difficult to oxidize than IAA decarboxylate slower. Thus, the radical cation of **8** (2-Me-5,6-diMeO-IAA) fragments ~100-fold faster than that of IAA (**1**) [25]. However, we have shown that only the radical cation of IAA decarboxylates (the deprotonated indolyl radical does not) [14], and the substituents which modify these effects also change the pK_a of the radical cation in a redox-controlled manner [9], so that compounds with faster decarboxylation rates of the radical cation have a smaller fraction of the cation in equilibrium at pH 7.4.

Thus, we have calculated that at this physiological pH, compounds **1, 3, 5**, and **6** should only show an approximately twofold variation in effective decarboxylation rates of the radical mixture [22], whereas **5** is much less cytotoxic than **1, 3**, or **6** when activated (Fig. 2). That there is no obvious link between decarboxylation rates and cytotoxicity is reinforced by the new data for the IPA derivatives **9** and **10**. Substitution by the exocyclic methyl group increases the fragmentation rate by at least 6-fold [25], but **9** and **10** were not cytotoxic in the absence or presence of HRP.

Discounting prodrug oxidation rates or radical decarboxylation rates as the key molecular properties controlling cytotoxicity in these analogues, we turn to a comparison of the cytotoxicity data with lipid peroxidation efficiency in liposomes of the indole/HRP combinations (Fig. 2). There is no obvious link between lipid peroxidation in liposomes and cytotoxicity in mammalian cells. Thus, while the two analogues most efficient in inducing lipid peroxidation (**3** and **6**, 2-Me-IAA and 2-Me-5-MeO-IAA) do show peroxidase-induced cytotoxicity, **1** and **2** (IAA and 1-Me-IAA), which are also cytotoxic when activated, are poor enhancers of the peroxidase-induced lipid peroxidation. The failure to detect lipid peroxidation in mammalian cells after indole/HRP treatments which are cytotoxic, and the lack of a significant effect of α-tocopherol on cytotoxicity with IAA/HRP (Fig. 3), are both suggestive that lipid peroxidation is not the main mechanism of cytotoxicity.

In apparent contradiction to this conclusion is the efficacy of the water-soluble analogue of α-tocopherol, Trolox, in completely preventing the cytotoxicity of IAA/HRP. However, we have shown that Trolox reacts directly with the radical obtained on indole oxidation with concomitant formation of the chromanoxyl radical [18]. The rate constant is such that at a concentration of 10 μM, the lifetime of the indolyl radical would be only ~2–3 msec. We ascribe the protective efficiency of Trolox not to any effect in chain-breaking of peroxidation reactions in the lipid, but rather to the interception of indole radicals in the aqueous, extracellular phase before damaging species can reach the cell (The hydrophilic antioxidant, ascorbate, was also effective in scavenging the indolyl radical and inhibiting lipid peroxidation in liposomes [18]; however, although it is possible to load V79 cells with ascorbate [35, 36], we found plating efficiencies were reduced.)

In work reported elsewhere [22], we showed that cytotoxicity remained or was reduced only a small amount if cells were incubated with the enzyme-free ultrafiltrate following reaction of the indole with HRP. Using the clonogenic assay, similar toxicity was observed compared to when the enzyme and indole were present. This implies that the toxic species is not a short-lived excited state generated during oxidation, but is more likely to be a relatively stable product from free radical reactions following one-electron oxidation. The present studies demonstrated that indole-derived species were bound in mammalian cells following treatment with IAA/HRP, and that in a model system of plasmid DNA incubated with the prodrugs and HRP, DNA damage resulted. Although the electrophoresis experiments were not analyzed quantitatively, the compounds which appeared to result in the most damage were those which exhibited cytotoxicity. Since reactivity towards other nucleophiles might parallel reactivity of an electrophilic intermediate towards DNA, this observation should not be taken to imply that DNA is the target critical for cytotoxicity.

The EPR spin-trapping experiments do provide a crude estimate of the rate constant for reaction of the skatolyl radical with DNA. The rate of reaction of DMPO with the skatolyl radical is not known, but if it reacts at about the same rate as, e.g. $^{\cdot}C(CH_3)_2OH$) [37], then our EPR results ($k_{DNA}/k_{DMPO} = 36 \pm 6$) suggest a rate of reaction between the skatolyl radical and DNA (k_{DNA}) close to the diffusion-controlled limit. However, since cytotoxicity was observed when the enzyme-free filtrates of indole/HRP incubations were incubated with cells [22], significant involvement of the skatolyl radical with DNA as a critical target can be ruled out.

Binding of nonradical intermediates in the peroxidation of IAA to histones [38] or nucleic acids [19, 20] has been reported previously, and attributed either to reaction of 3-methylene-2-oxoindole with sulphydryl residues, or to an electronically excited state or indole-3-aldehyde, respectively. As our cell-free incubation experiments show that the cytotoxic agent is long-lived, we can probably rule out the latter. Oxidation of IAA by HRP yields 3-methylene-2-oxoindole [3, 4, 6], but it might be expected that 2-methyl substitution would influence the formation of this or analogous products, and the 2-methyl derivative was the most cytotoxic of the analogues studied.

In conclusion, our results demonstrate significant cytotoxicity of IAA derivatives, in combination with peroxidase. However, there was no apparent correlation between lipid peroxidation and cytotoxicity, and extensive efforts to detect lipid peroxidation in cells were unsuccessful. Evidence for binding of indole-related products to whole cells and formation of DNA adducts, following the indoleacetic acid/peroxidase treatment, was also obtained. The addition of skatolyl-type radicals to DNA was demonstrated by EPR with competitive spin-trapping. However, in the cell system the skatolyl radicals are generated by extracellular peroxidase, and easily scavenged by inhibition by a hydrophilic antioxidant; it is unlikely that this reactive and short-lived radical would diffuse to the cell nucleus to cause damage to the nucleic acid. More convincingly, stable products of peroxidase-catalyzed decomposition are cytotoxic. Thus, our data point to unidentified (probably electrophilic) oxidation products, reactive towards intracellular targets, being involved in cytotoxicity of the IAA/HRP combination, rather than direct attack of free radicals, excited states, or membrane lipid peroxidation, as might be concluded from the earlier studies.

Further work is needed to identify the cytotoxic oxidation product and the cellular target. This may enable a

more rational approach to the identification of compounds which might have therapeutic potential. Nevertheless, the results presented show the cytotoxic potential of drug/peroxidase combinations, and these indoles might be useful as the basis for novel prodrugs for use in cancer therapy involving antibody targeting of peroxidase.

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