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5-Fluoroindole-3-acetic acid: a prodrug activated by a peroxidase with potential for use in targeted cancer therapy

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Abstract

Indole-3-acetic acid and some derivatives are oxidized by horseradish peroxidase, forming a radical-cation that rapidly fragments (eliminating CO_2) to form cytotoxic products. No toxicity is seen when either indole-3-acetic acid or horseradish peroxidase is incubated alone at concentrations that together form potent cytotoxins. Unexpectedly, 5-fluoroindole-3-acetic acid, which is oxidized by horseradish peroxidase compound I 10-fold more slowly than indole-3-acetic acid, is much more cytotoxic towards V79 hamster fibroblasts in the presence of peroxidase than the unsubstituted indole. The fluorinated prodrug/peroxidase combination also shows potent cytotoxic activity in human and rodent tumor cell lines. Cytotoxicity is thought to arise in part from the formation of 3-methylene-2-oxindole (or analogues) that can conjugate with thiols and probably DNA or other biological nucleophiles. Levels of the fluorinated prodrug in the murine carcinoma NT after intraperitoneal administration of 50 mg/kg were about 200 μM. Although these were 4–5-fold lower than plasma levels (which reached 1 mM), the integrated area under the concentration/time curve in tumors over 2 hr was ~20 mM min, almost double the exposure needed to achieve ~90–99% cell kill in human MCF7 breast or HT29 colon tumor cell lines and CaNT murine cells *in vitro*, although the human bladder T24 carcinoma cell line was more resistant. The high cytotoxicity of 5-fluoroindole-3-acetic acid after oxidative activation suggests its further evaluation as a prodrug for targeted cancer therapy involving antibody-, polymer-, or gene-directed delivery of horseradish peroxidase or similar activating enzymes.

Keywords: Indole-3-acetic acid; 5-Fluoroindole-3-acetic acid; Peroxidase; Horseradish peroxidase; Prodrug; 3-Methylene-2-oxindole

1. Introduction

Approaches to cancer therapy involving prodrugs activated by tumor-targeted enzymes, such as in the ADEPT strategy [1], have utilized many different prodrugs. Examples include alkylating mustards masked either by conjugation (activated by carboxypeptidase), or deactivated nitroarene moieties (activated by nitroreductase) [2]. We have identified an alternative prodrug/enzyme combination that is worthy of exploration because of the known low toxicity of the prototype prodrug and the potential of the activating enzyme to be modified to reduce immunological reactions. Indole-3-acetic acid (IAA) (Fig. 1, **1**), a plant growth hormone, is oxidized by horseradish peroxidase (HRP) to form products cytotoxic to mammalian cells [3]. The initiating hypothesis was based on the possibility that IAA/HRP would initiate lipid peroxidation, as found in liposome models [3–6]. The free radical formed on one-electron oxidation of IAA (the indolyl radical-cation, Fig. 1, **2**) was known to fragment in ~40 μs, releasing CO_2 to form the skatolyl radical **3**. In the presence of oxygen this forms the skatole peroxyl radical **4**, the presumed reactive intermediate in lipid peroxidation [7]. However, cytotoxicity was demonstrated in experiments in which IAA was oxidized either by HRP or by radiolysis and the stable products then added to cells [8], indicating the short-lived peroxyl radical was not itself the damaging species. Lipid peroxidation was not detectable in mammalian cells after cytotoxic treatments, also pointing to other mechanisms of cytotoxicity [9]. Toxicity has been measured in Chinese hamster lung fibroblast V79 cells with a range of different IAA analogues, and differences

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Abbreviations: BSO, L-buthionine sulphoximine; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's modified medium; FCS, fetal calf serum; FIAA, 5-fluoroindole-3-acetic acid; FMOI, 5-fluoro-3-methylene-2-oxindole; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; MOI, 3-methylene-2-oxindole; SMEM, spinner modified EMEM.

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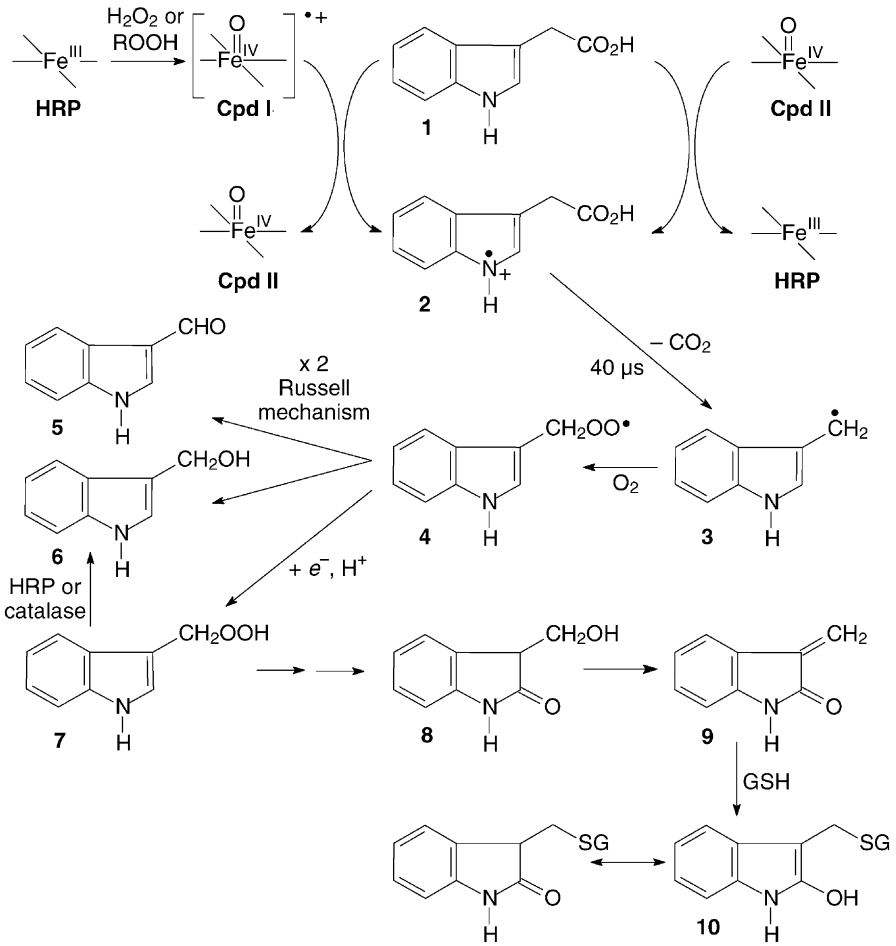

Fig. 1. Outline of the mechanistic pathways involved in the activation of IAA derivatives by HRP to form potentially cytotoxic nucleophiles, methyleneoxindoles. Added hydrogen peroxide is not required. Trace peroxides in media (or produced by cells) can initiate the reaction, which then proceeds through a 'branched chain mechanism' [11] since fragmentation of the radical-cation **2** leads to the hydroperoxide **7**, itself capable of forming Cpd I from HRP.

in toxicity were observed with different substituents. Damage to liposome model systems and plasmid DNA was seen [8], but the cause of cellular toxicity remains unclear.

HRP compounds I and II (Cpds I and II, oxidation states shown in Fig. 1) are key oxidizing intermediates in the action of the peroxidase [10–12] (see Fig. 1). These oxidize IAA in the absence of hydrogen peroxide to the indolyl radical-cation **2**, the precursor of the carbon-centered skatolyl radical **3** [13,14]. This radical can abstract a hydrogen atom from DNA in anoxia [8], or react with oxygen to form the skatole hydroperoxyl radical **4**. This by further steps (see Fig. 1) leads to the major products: indole-3-aldehyde (**5**), indole-3-carbinol (**6**), skatole hydroperoxide (**7**), oxindole-3-carbinol (**8**) and 3-methylene-2-oxindole (MOI, **9**) [11,15]. The latter is a known product of the IAA/HRP reaction [16], and is a candidate for the putative toxic species in aerobic systems due to its reactivity towards cellular nucleophiles, such as thiols or DNA, as illustrated in the formation of the GSH adduct, Fig. 1 (**10**) [9,17–19].

The rates of oxidation of simple amines and phenols by the key peroxidase intermediates HRP Cpds I and II show a marked dependence on substituents that modify the redox properties [11,20]. This was also found to be the case with substituted indole-3-acetic acids [21,22]. Substitution by electron-withdrawing halogen moieties, such as fluorine, is expected to deactivate indole-3-acetic acids towards oxidation by HRP: redox relationships for oxidation of IAA derivatives by HRP Cpd I using either Hammett sigma substituent parameters or reduction potentials of the radicals were established [21,22]. (Oxidation of most substrates by Cpd II is usually several-fold more slowly than Cpd I, although parallel redox relationships are observed.) However, factors other than the rates of oxidation or decarboxylation by HRP may control activity. We show here that 5-fluoroindole-3-acetic acid (FIAA), is indeed oxidized an order of magnitude more slowly than IAA by HRP Cpd I, but is much more toxic when activated, indicating that the effects of substituents on the reactivity of products as well as the rate of activation has to be considered.

2. Materials and methods

2.1. Materials

HRP type IV-A, FIAA, IAA, L-buthionine sulphoximine (BSO), fetal calf serum (FCS), Eagle's modified medium (EMEM) and spinner modified EMEM (SMEM), non-essential amino acids, penicillin, streptomycin, L-glutamine, trypsin and phenol-red free Hanks' balanced salt solution were obtained from Sigma. Monobromobimane was obtained from Molecular Probes. Dulbecco's modified Eagle's medium (DMEM) was obtained from Life Technologies. Other chemicals were from Merck, AnalaR grade.

2.2. Cells

Chinese hamster V79 lung fibroblast, HT29 human colon carcinoma, MCF7 human breast carcinoma, CaNT mouse carcinoma, and T24 human bladder carcinoma cells were obtained from the European Collection of Cell Cultures. V79 cells were maintained in EMEM supplemented with 10% FCS, or SMEM supplemented with 7.5% FCS. CaNT cells were maintained as attached monolayers in EMEM with 10% FCS, HT29 cells and MCF7 cells similarly in EMEM supplemented with 10% FCS and 1% non-essential amino acids, and T24 cells similarly in DMEM supplemented with 10% FCS. All media were supplemented with 2 mM L-glutamine, 100 unit/mL penicillin and 100 μg/mL streptomycin. All cells were subcultured by trypsin removal of the cells (1 × 0.5% porcine trypsin, 0.2% EDTA).

2.3. Measurements of cytotoxicity

Indole stock solutions were prepared daily in 1 or 10% ethanol and protected from light. The pH was adjusted to 7.4 for high final concentrations of indoles. For cytotoxicity experiments, V79 cells were allowed to attach, from spinner culture, for at least 1 hr before administration of the drug; HT29, MCF7 and T24 cells were allowed to attach for at least 4 hr, and CaNT cells overnight (16 hr) following trypsin removal.

Cell survival experiments were carried out as previously described [8]. Attached cells on Petri dishes (200–20,000 cells) were treated with 2 mL indoles (50 or 100 μM) and HRP (1.2 μg/mL) in phenol red free Hanks' balanced salt solution, then washed with 2 mL Hanks', and left to form colonies in EMEM for 7 days. After growth, colonies were fixed with 75% methanol and stained with 1% (w/v) crystal violet. Colonies containing >50 cells were counted and surviving fractions (SF) calculated relative to untreated controls.

2.4. Effects on intracellular glutathione

Cellular glutathione (GSH) depletion was measured by plating 10^6 cells in phenol red free DMEM. The cells were left to attach for 1 hr, then 50 μM IAA or FIAA added in phenol red free Hanks' solution, with or without 1.2 μg/mL HRP. Samples were taken from 0 to 2 hr. Cells were washed with 4 × 2 mL Hanks' solution and scraped in 1 mL 50 mM perchloric acid/1 mM EDTA before freezing at −20°. Duplicate samples were thawed and solid matter removed by centrifugation. Supernatants were analyzed for GSH content after derivatization with monobromobimane (MBB) [23]. Samples (0.4 mL) were mixed with mercaptoethanol (25 μL, 100 μM), MBB (25 μL, 10 mM) and Tris–HCl (250 μL, 2 M containing 1 mM EDTA) for 15 min in the dark. The samples were then acidified with HCl (50 μL, 6 M) and interfering substances removed by extraction with 0.5 mL dichloromethane, retaining the sample in the aqueous phase. HPLC involved a 250 mm × 4 mm Hypersil 5ODS column eluting at a flow rate of 2 mL/min with a gradient of $NH_4H_2PO_4$ (40 mM)/H_3PO_4 (10 mM)/1-octanesulphonic acid (5 mM) and 10–40% acetonitrile/water (75% (v/v)) over 10 min. Detection was by fluorescence (Perkin-Elmer LS40 detector, excitation 398 nm, emission 476 nm).

The effect of GSH depletion in V79 cells was carried out by treating 3×10^5 V79 cells/mL in SMEM with 0.1 mM BSO overnight (16 hr). The cells were counted the following day and plated on Petri dishes in phenol red free DMEM for 1 hr prior to drug treatment. The cytotoxicity of 50 μM FIAA or IAA was then measured as already described and compared to non-GSH depleted cells. GSH depletion was checked by washing treated cells in Hanks' solution, lysing in 50 mM PCA/1 mM EDTA and storing at −20° for HPLC analysis.

2.5. Analysis of products

The products of oxidation of FIAA with HRP, and reactivity of FMOI with thiols were measured by oxidizing 0.1 mM FIAA with 10 μg/mL HRP in Hanks' for 2 hr. Excess GSH or cysteine (1 mM) was added and the loss of FMOI demonstrated by HPLC. Compounds were eluted on a reversed phase RPB column (100 mm × 3.2 mm) with 50 mM ammonium acetate, pH 5.1 (A) and methanol (B) with a gradient of 20–60% B in 8 min at 0.9 mL/min. Detection was by a Waters photo diode array detector extracting at 250 nm. Mass spectral analysis (LCMS) was carried out using a Waters ThermaBeam mass detector, operating in scan mode over an appropriate mass range using electron impact ionization.

2.6. Kinetics of reaction with HRP compound I

The rate of reaction of FIAA with HRP compound I, formed from a 1 s premixing of equimolar HRP and hydrogen peroxide (0.47 μM), was carried out as previously described [21] using a Hi-Tech SF-61 DX2 double mixing stopped-flow spectrophotometer equipped with a xenon lamp. Formation of HRP compound II was detected

at 411 nm at 25° in 10 mM phosphate buffer pH 7 with 50 mM potassium bromide.

2.7. Distribution of FIAA in murine tissues

FIAA (5 mg/mL) in 2% (v/v) ethanol/water was adjusted to pH 7.4 with NaOH and injected i.p. (50 mg/kg) in female CBA mice bearing the CaNT tumor. The mice were sacrificed up to 2 hr after drug administration by decapitation, and the blood (heparin-coated tubes) and tissues removed and immediately placed on ice. The whole blood was spun down and the plasma stored at −20°. Tissue samples were weighed and homogenized in four to nine volumes of ice-cold water added. The homogenized tissue was stored at −20° before HPLC analysis.

For HPLC analysis, plasma (50 μL) was mixed with IAA internal standard (130 μM, 25 μL) and the protein precipitated with acetonitrile (50 μL). The samples were spun down and the supernatant injected directly for HPLC analysis. For tissue levels, samples (250 μL) were mixed with IAA (130 μM, 25 μL) and precipitated with 250 μL acetonitrile for direct injection for HPLC. HPLC analysis was carried out with a Hypersil 5ODS 125 mm × 4.6 mm column eluting with A: 75% acetonitrile and B: 20 mM ammonium acetate (pH 5.1) with a gradient of 15–70% A in 10 min at 2 mL/min. Detection was at 290 nm using a Waters 486 variable wavelength detector. Calibration curves were linear up to 1 mM FIAA ($R = 0.9998$) and recovery of added IAA was >95%.

3. Results

3.1. Differential cytotoxicity of FIAA with/without HRP

Toxicity towards V79 cells of 50 μM FIAA or IAA with 1.2 μg/mL HRP was measured after 0–2 hr exposure (Fig. 2). No surviving cells (SF $< 10^{-4}$) could be detected with 100 μM FIAA + 1.2 μg/mL HRP after treatment for only 1.5 hr. Treatment with IAA, FIAA or HRP alone at these concentrations had no detectable effect on cell survival.

In other experiments, V79 cells were depleted of GSH by treatment with BSO overnight. Cellular GSH levels were measured and were <0.1% of initial values. This resulted in enhanced cytotoxicity of 50 μM IAA and 1.2 μg/mL HRP. GSH depletion also enhanced the cytotoxicity of the FIAA/HRP combination at treatment times up to 1.5 hr, although at 2 hr the effect of BSO treatment was not evident (Fig. 2).

Cytotoxicity of the FIAA/HRP combination was also analyzed in a range of human and rodent tumor cell lines: human breast carcinoma MCF7 cells, human colon carcinoma HT29 cells, human bladder carcinoma T24 cells, or mouse carcinoma NT cells. Since these were more resistant to the treatment than V79 cells, 100 μM concentrations of FIAA were used. The relationships between survival and exposure time were qualitatively similar to those shown in Fig. 2; Table 1 summarizes the cytotoxic responses, and compares them with measurements or published values of the intracellular GSH levels. No toxicity was detectable with any of the cell lines with HRP or prodrug alone at these drug concentrations and times.

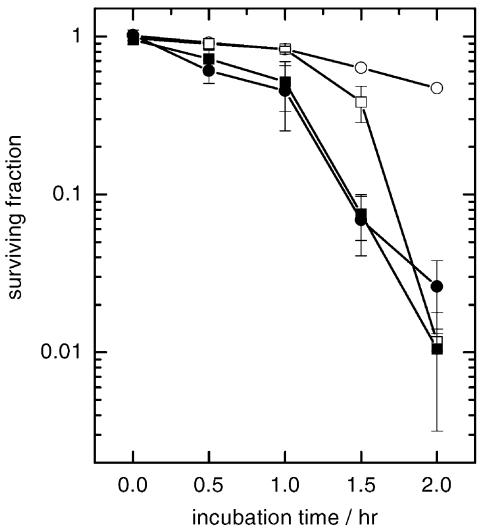

Fig. 2. Surviving fractions of V79 cells treated for up to 2 hr with 50 μM IAA (○) or FIAA (□) with 1.2 μg/mL HRP, and similar experiments with cells depleted of GSH by treatment with BSO, exposed to IAA (●) or FIAA (■). Data shown are the means and standard errors of three independent experiments (errors not shown where less than symbol size).

The effects of varying concentrations of FIAA and HRP were investigated using V79 or CaNT cells exposed for 5 hr (Fig. 3). The concentrations of FIAA required to kill 50% of the cells (IC_{50}) in the presence of 1.2 μg/mL HRP were estimated as 6 μM with V79 cells, or 17 μM with CaNT cells. In the absence of HRP the IC_{50} of FIAA was 2.5 or 5 mM with V79 or CaNT cells, respectively, after 5 hr exposure (Fig. 3(A)). A ∼400-fold differential (in concentration terms for equal cytotoxic effect) was thus demonstrated between indole toxicity with or without HRP with V79 cells, and a ∼300-fold differential for CaNT cells.

Table 1
Cytotoxicity of FIAA (100 μM) with HRP (1.2 μg/mL) towards rodent and human cells after 2 hr exposure

Cell line	Surviving fraction at 2 hr	GSH/NPSH (μmol/g protein)
V79	$<10^{-4}$	20[a]
MCF7	0.0054 ± 0.0003	98[b]
HT29	0.010 ± 0.005	29[c]
CaNT	0.064 ± 0.040	Not known
T24	0.185 ± 0.060	16[d]

[a] This work.
[b] [24].
[c] [25].
[d] [26].

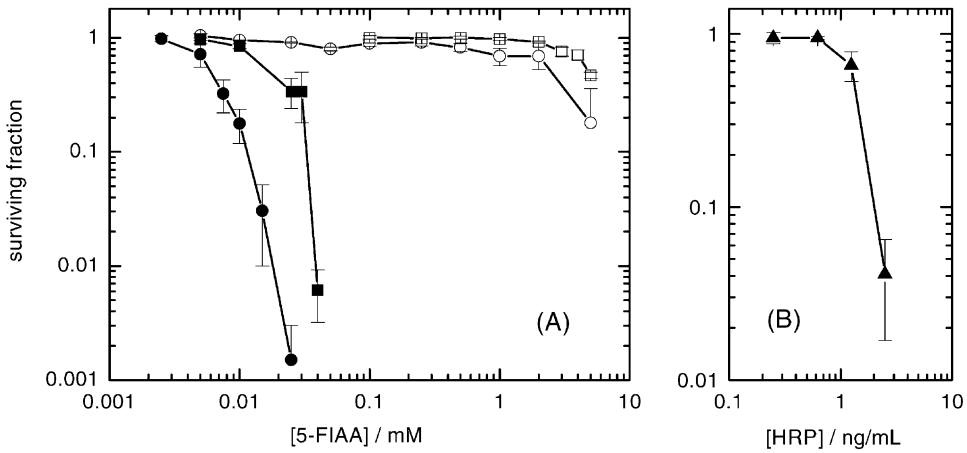

Fig. 3. (A) Surviving fractions after exposure of V79 cells for 5 hr to FIAA without (○) or with (●) 1.2 μg/mL HRP, and similar experiments using mouse carcinoma NT cells without (□) or with (■) HRP. (B) Surviving fractions (▲) of V79 cells following 5 hr exposure to 50 μM FIAA and varying HRP concentrations (2 ng/mL ≈ 50 pM). Data shown are the mean and standard errors of three independent experiments.

After 5 hr, 50% cell kill was shown with only ∼1.5 ng/mL HRP and 50 μM FIAA in V79 cells (Fig. 3(B)).

3.2. Reaction of an oxidation product with GSH

The putative toxic product of oxidation, 5-fluoro-3-methylene-2-oxindole (FMOI), was expected to react with thiols (see Fig. 1). HRP oxidation of FIAA produced a product identified as FMOI by its mass spectrum after HPLC separation (Fig. 4, peak 5), which was lost after incubation in excess GSH with the formation of a more polar compound (Fig. 4, peak 2). This is believed to be a conjugate with the thiol. Similar results were also seen with cysteine. (Using electron impact ionization, mass spectral confirmation of the mass of this product was not possible; only the fragment corresponding to the oxindole residue was observed.)

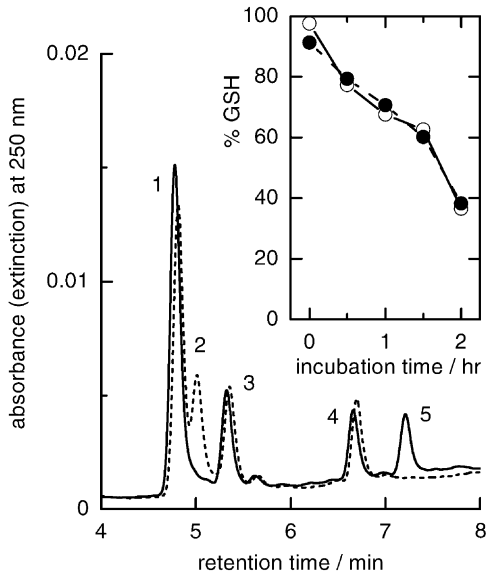

Fig. 4. HPLC chromatogram (—) showing separation of the products of oxidation of FIAA (peak 1) by HRP: 5-fluoroindole-3-carbinol (peak 3), 5-fluoroindole-3-aldehyde (peak 4) and 5-fluoro-3-methylene-2-oxindole (peak 5). The chromatogram (- - -) after addition of GSH shows loss of peak 5 and production of a thiol conjugate (peak 2). The insert shows depletion of GSH following treatment of V79 cells with 50 μM IAA (○) or FIAA (●) and 1.2 μg/mL HRP. Data shown are the means of duplicate experiments.

In order to investigate the role that GSH depletion may have in cellular toxicity, the GSH concentrations were measured in V79 cells treated with 50 μM IAA or FIAA and 1.2 μg/mL HRP for up to 2 hr. Intracellular GSH levels decreased with time, with a maximum loss of ∼65% of initial (control) levels of GSH after 2 hr (Fig. 4). No effect on GSH levels was seen with either indole or HRP alone.

3.3. Relative oxidation rates of FIAA and IAA

FIAA was found to react with HRP compound I with a rate constant of $(3.82 \pm 0.08) \times 10^2\ M^{-1}\ s^{-1}$ at pH 7. This is an order of magnitude lower than reaction with IAA, $(3.79 \pm 0.07) \times 10^3\ M^{-1}\ s^{-1}$ [22]. Despite the much lower rate of oxidation, major products of FIAA oxidation by HRP corresponded to those formed with IAA: 5-fluoroindole-3-carbinol, 5-fluoro-2-oxindole-3-carbinol and 5-fluoro-3-methylene-2-oxindole were detected by UV–VIS absorbance and mass spectrometry (Fig. 4). The absorption spectra of the products were very similar to those reported for IAA oxidation [15] with corresponding shifts in the mass spectra and HPLC retention.

3.4. Distribution of FIAA in tumor-bearing mice

Fig. 5 shows levels of FIAA in several tissues after i.p. administration of 50 mg/kg FIAA. No metabolites of FIAA were seen in any of the samples. FIAA was cleared quite slowly from plasma, with a half-life of 1.5 hr. Tissue concentrations were considerably lower than plasma. The

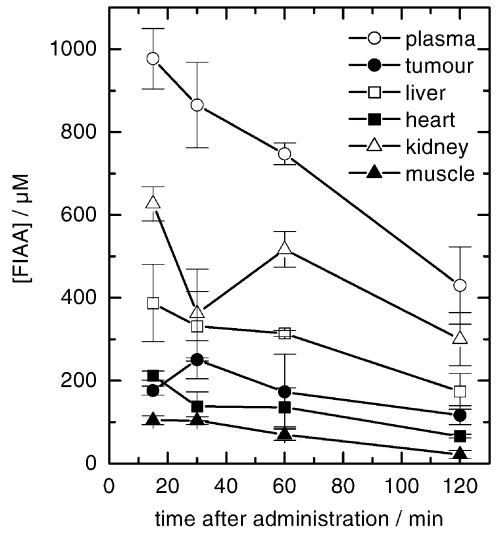

Fig. 5. Distribution of FIAA in female CBA mice bearing the carcinoma NT after administration of 50 mg/kg i.p. Data shown are the mean and standard errors of three experiments.

areas under the concentration/time curves over 2 hr were computed (mM min): plasma, 81; tumor, 20; liver, 33; heart, 14; kidney, 50; skeletal muscle, 8.

4. Discussion

4.1. Effect of fluorine substitution on oxidation by HRP and the mechanisms of cytotoxicity

Fluorine substitution introduces an electron-withdrawing group, and from the well-established redox dependence of the rate constant for reaction of HRP Cpd I [21,22,27], FIAA was predicted to react with HRP much more slowly than IAA itself. This was confirmed by stopped-flow spectrophotometry, HRP Cpd I reacting with FIAA ~10-fold more slowly than IAA at pH 7. These rate constants for reaction with Cpd I are mirrored by the relative rates of loss of parent indole and formation of products upon treatment with HRP (data not shown). V79 cells treated with 5-^{3}H-IAA and HRP showed intracellular accumulation of ^{3}H, especially in the nuclear fraction, compared to controls without HRP [8,9]. This indicates that an oxidation product is binding to cellular and nuclear components. It is unlikely that the oxidation products of FIAA are qualitatively different to those of IAA, as similar products were detected by HPLC and identified by UV–VIS spectrophotometry and MS detection following HRP oxidation. If the product that is binding to cellular components is MOI or FMOI (electrophiles susceptible to nucleophilic attack) one would expect fluorine substitution, by its electron withdrawing nature, to enhance the electrophilic properties of the methylene group. MOI has been shown to bind to DNA histones [19], and RNA [28] in plants and DNA plasmids [8].

The toxicity of FIAA/HRP in these experiments is thought to be largely associated with relatively stable products rather than free-radical intermediates. Toxicity towards V79 cells was seen after treating cells with the stable products resulting from oxidation of IAA by HRP treatment [3]. Addition of excess GSH to these products significantly reduced cytotoxicity [3], implying that potentially cytotoxic product had reacted with the thiol, removing its ability to react with biologically important nucleophiles. FMOI was shown by HPLC to react with GSH and cysteine to form a more polar compound and it is the most likely candidate for the putative cytotoxin. No reaction of FMOI was seen with DNA free bases or nucleotides, or other amino acids, but protein thiols are expected to be reactive. After treating V79 cells with ^{3}H-labeled IAA, activity is seen in washed cell fractions after lysis [8], suggesting that non-cytoplasmic conjugation is occurring and that GSH is not the main target of MOI. Reactivity with different cellular components will depend upon the rate constants for reaction with each target. These are presently unknown due to difficulty in isolating pure MOI or FMOI in aqueous solutions, and further work is required.

Oxidation of FIAA by HRP is thought to occur extracellularly as HRP does not enter cells readily; the indole equilibrates across the cell membrane in 30 min in V79 cells, as shown by measurements similar to those described earlier with IAA [8] (data not shown). The products of the reaction, especially FMOI, should cross the cytoplasmic membrane easily, being uncharged and small. Diffusion to the nucleus may be impeded by cytoplasmic GSH. Experiments involving GSH depletion showed that IAA toxicity is increased by loss of GSH, suggesting the GSH is protective with this prodrug. However, the effect was smaller with FIAA.

4.2. Relative cytotoxicity of IAA and FIAA and variations between cell lines

FIAA was shown to be much more cytotoxic than IAA when activated by HRP, using 50 μM prodrug (Fig. 2) and using 100 μM prodrug (Table 1) (previously published data for IAA [3] showed a SF of 0.015 with 100 μM IAA + 1.2 μg/mL HRP after 2 hr). This is not consistent with the relative reactivities of the key oxidizing intermediate, Cpd I (also expected to be representative of Cpd II) and points to the importance of substitution on the reactivities of products, such as the methyleneoxindole (Fig. 1, **9** and analogues).

The variation in cytotoxicity of FIAA/HRP with the various cell lines may be compared with reported differences in cellular GSH levels (Table 1). The prodrug/HRP combination was cytotoxic in the order T24 < CaNT < HT29 < MCF7 < V79 cells. Cellular GSH levels measured in different studies are not in the same ranking order, although protein levels may differ and cytoplasmic GSH

concentrations cannot be inferred directly. However, T24 bladder carcinoma cells are known to be chemoresistant [29]. Other possible complications of interpretation, such as the compartmentation of GSH [30], have been discussed in a study of the links between cellular GSH and ADEPT involving a masked alkylating mustard [31]. Treatment with either indole together with HRP results in approximately the same extent of thiol loss (Fig. 4) in spite of FIAA being much more toxic than IAA in the presence of HRP, suggesting that other cellular damage is occurring that is associated with toxicity.

4.3. Tissue distribution of FIAA and the potential cytotoxicity of FIAA/HRP in vivo

Fluorine substitution in the 5-position may block the usual IAA hydroxylation site during metabolism, explaining the absence of detectable metabolites; it is possible that most FIAA is excreted unchanged, although this needs to be confirmed. The van der Waals radius of fluorine is only about 13% greater than that of hydrogen, and binding of FIAA to any receptor sites should be broadly similar to IAA. Hence, it is likely that the doses of IAA or FIAA tolerated by animals might be not dissimilar. The dose of FIAA used in the present study (50 mg/kg) equals 0.26 mmol/kg. IAA can be administered to mice at 2 mmol/kg,[1] and IAA is known to be tolerated by humans in doses of 100 mg/kg (0.57 mmol/kg) [32]. The present work shows that the lower dose of 50 mg (0.26 mmol/kg) FIAA administered to mice results in tumor levels of about 200 μM FIAA. Such levels are 5- to 10-fold higher than those resulting in high levels of cytotoxicity in hamster fibroblasts or murine tumor cells after 5 hr exposure in the presence of HRP (Fig. 3).

Possibly a more realistic comparison is between the area under the concentration/time curve (AUC) in the tumor and the exposure needed for cell kill *in vitro*. After 2 hr, this is 20 mM min in the murine tumor (Fig. 5). The exposure corresponding to the treatment *in vitro* for the conditions described in Table 1 is almost half this level (12 mM min), after which cell kill is ∼99 or ∼90% in human tumor breast (MCF7) or colon (HT29) cell lines, respectively. However, the chemoresistant T24 bladder carcinoma cell line is less sensitive to FIAA/HRP.

Targeting of HRP to a tumor by antibody-, polymer-, or gene-directed methods would allow tumor specificity to be achieved, since mammalian peroxidases are ineffective in activating the prodrug. Fig. 3(B) shows that significant cytotoxicity can be achieved with HRP levels much smaller than 1.2 μg/mL used in most of the experiments. Preliminary experiments (data not shown) by R.B. Pedley and S. Cooke (University College London/Royal Free Hospital) have demonstrated that it is possible to conjugate HRP to the tumor-targeting anti-CEA antibody used in ADEPT studies. Obviously, to predict the likely magnitude and differential effects on tumors relative to normal tissues *in vivo* in such a strategy requires much further work. However, the effects of variations in concentrations of both prodrug and enzyme reported here provide a basis for the studies needed.

The alternative gene-directed (GDEPT) strategy is being explored in this institute. IAA has recently been shown to be selectively toxic towards T24 human bladder carcinoma cells transfected with a mammalian expression vector containing the HRP cDNA [33].

Although the details of the mechanism of cytotoxicity of the combination of FIAA and HRP remain uncertain, the formation of the electrophilic methyleneoxindole oxidation product and reactivity towards a nucleophile has been demonstrated. More importantly, the cytotoxic effectiveness of the fluorinated prodrug is significantly higher than that of the parent IAA. Further work to understand the enhanced activity conferred by fluorine substitution, by extending this work to other halogenated or alternative electron-withdrawing substituents, is merited. Direct measurements of the cytotoxicity and chemical reactivity of the putative (oxindole) cytotoxins are also needed. However, the present study has clearly demonstrated that the combination of a halogenated indole acetic acid and HRP has the properties required to justify further evaluation of its potential in targeted cancer therapy *in vivo*.

Acknowledgments

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