Reactivity toward Thiols and Cytotoxicity of 3-Methylene-2-oxindoles, Cytotoxins from Indole-3-acetic Acids, on Activation by Peroxidases

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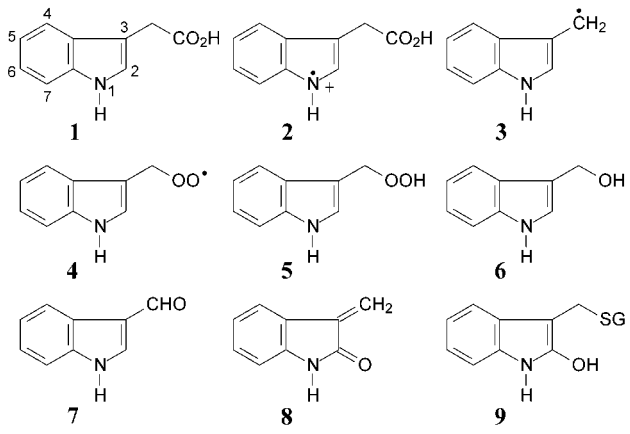
Oxidation of indole-3-acetic acid and its derivatives by peroxidases such as that from horseradish produces many products, including 3-methylene-2-oxindoles. These have long been associated with biological activity, but their reactivity has not been characterized. We have previously demonstrated the potential value of substituted indole acetic acids and horseradish peroxidase as the basis for targeted cancer therapy, since the compounds are of low cytotoxicity until oxidized, when high cytotoxicity is observed; the combination of prodrug and enzyme depletes intracellular thiols. In this study, 3-methylene-2-oxindole and derivatives substituted in the 4-, 5-, or 6-position with methyl, F, or Cl have been synthesized and their reactivity toward representative thiol nucleophiles (glutathione, cysteine, and a cysteinyl peptide) measured using stopped-flow kinetic spectrophotometry. Rate constants were in the range ∼2 × 10^3 to 2 × 10^4 M^{-1} s^{-1} at pH 7.4, 25 °C, implying a lifetime of a few tens of milliseconds for these methylene oxindoles in the cellular environment and diffusion distances of a few micrometers. As expected, halogen substitution decreased the rate of production of the methylene oxindoles on treatment of horseradish peroxidase. The cytotoxicities of the compounds were measured using Chinese hamster V79 fibroblast-like cells in vitro. The halogen-substituted derivatives were much more cytotoxic than the 5-methyl analogue or the parent (unsubstituted) compound, consistent with the trends in rate constant for reaction with the thiols. The results show that the cytotoxic response in the prodrug (indole acetic acid) and enzyme (horseradish peroxidase) system reflects the reactivity of methylene oxindoles toward nucleophiles much more than the rate of generation of the oxindoles, and helps explain the possible advantages of 5-fluoroindole-3-acetic acid compared to IAA as a lead compound for investigation in targeted cancer therapy.

Introduction

Indole-3-acetic acid (IAA,[1] Scheme 1, **1**) is a plant growth hormone (auxin) (*1*) that is oxidatively degraded by peroxidases; numerous studies utilizing horseradish peroxidase (HRP) have been reported, meriting a separate chapter in a monograph on heme peroxidases (*2*). Unusually, the usual cofactor for oxidations catalyzed by HRP, hydrogen peroxide, is not required. One explanation may be that oxidation involves as a first step the formation of a radical cation (Scheme 1, **2**) that fragments in microseconds (*3*) to release CO_2 from the side chain, forming a carbon-centered skatolyl radical (Scheme 1, **3**). This radical reacts rapidly with oxygen to form a peroxyl radical (**4**) that is reduced by IAA or cellular reductants to the hydroperoxide (**5**), which may substitute for H_2O_2 in forming the reactive peroxidase intermediate, compound I from HRP. (Trace peroxides in biological media may also be sufficient to initiate the catalytic cycle.)

The alcohol (**6**) and aldehyde (**7**) may also be formed via the peroxyl radical (**4**), but most interest in respect of the biological activity of IAA in plants has focused on a subsequent degradation product, 3-methylene-2-oxindole (**8**) (*4*−*13*). Our interest in the IAA/HRP combination arose from the possible application of IAA as a prodrug in cancer therapy, with HRP generating a cytotoxin (*14*−*17*). Targeting the activating enzyme, using, e.g., antibody-directed [ADEPT (*18*)] or gene-directed [GDEPT (*19*)] approaches, could provide a basis by which the combination of IAA or an analogue with HRP or another suitable peroxidase could form an alternative prodrug/enzyme

Scheme 1. Structures of Indole-3-acetic Acid and Some of the Products Formed on Reaction with Horseradish Peroxidase



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[1] Abbreviations: CysSH, cysteine; EMEM, Eagle's modified medium; GSH, glutathione; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; MOI, 3-methylene-2-oxindole; SMEM, spinner-modified EMEM.

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strategy for cancer therapy, meriting comparison with those already being evaluated (*20*, *21*). Demonstration of the potential application of transfection of the HRP gene has been reported (*22*, *23*). One strategy toward tumor selectivity with gene therapy seeks to place the HRP gene under the control of hypoxia-responsive promoters (*23*), exploiting the different oxygen status of tumors and normal tissues (*24*). Mammalian peroxidases catalyze the formation of oxidized products from IAA much less efficiently than HRP, and hydrogen peroxide is an essential cofactor with the mammalian peroxidases (*25*, *26*) and several other fungal and plant peroxidases (*27*) but not HRP. [The reactivities of the enzyme intermediates, compounds I and II, toward some indoles are, however, lower for HRP than for myeloperoxidase or lactoperoxidase (*28*).] Although the skatolyl-type radical from the easily oxidized 2-methyl IAA derivative was detected by spin trapping in myeloperoxidase-rich human leukemia HL-60 cells without added HRP, very high concentrations of the prodrug were needed. Further, these high concentrations resulted in much less cytotoxicity in this cell line than observed using 3 orders of magnitude lower concentrations of the prodrugs with HRP in fibroblasts and cell lines from solid human or murine tumors (*14*, *17*).

While our original hypothesis was based on enhanced oxidative stress from the peroxyl radical intermediate (*29*), involving (for example) lipid peroxidation (*14*−*16*), it soon became evident that the biologically active intermediate was much longer-lived (*14*−*16*). Our interest naturally turned to the methylene oxindole (**8**) as a putative cytotoxin in mammalian cells, but it is poorly characterized. Although it is known to be reactive toward thiols (*7*) or histones (*8*), and depletion of intracellular thiols after exposure to IAA/HRP has been demonstrated (*17*), reactivity has not been quantified. In addition to the mechanistic relevance, it is important to know the rate constants of reaction with key 'sinks' for the reactive cytotoxin, as these will determine the diffusion distance and hence the potential for 'bystander' effects. An additional reason for characterizing some of the properties of methylene oxindoles was our unexpected observation that substitution of IAA with fluorine in the 5-position significantly enhanced the cytotoxicity of the combination with HRP compared to IAA (*17*). This was despite the expected—and found—*decrease* in the ease of oxidation by the peroxidase intermediates (*30*, *31*) conferred by the electron-withdrawing substituent, since peroxidase-catalyzed oxidations have remarkably high redox dependencies (*2*, *32*, *33*).

In this study, we have synthesized 3-methylene-2-oxindole (MOI, Scheme 1, **8**) and five derivatives substituted with methyl, F, or Cl in the benzenoid ring, to vary the redox properties. Rate constants for reaction of MOI or derivatives with glutathione (GSH) and cysteine (CysSH) have been measured using stopped-flow kinetic spectrophotometry, the cytotoxicities of the compounds have been measured using mammalian fibroblasts, and the production of MOI or derivatives by treatment of the corresponding IAA derivatives with HRP has been compared. The results help explain the unexpected high cytotoxicity of some halogenated IAA derivatives when activated by HRP.

Experimental Procedures

Materials. All chemicals unless otherwise stated were obtained from Sigma, U.K. The peptide Lys-Cys-Thr-Cys-Cys-Ala (trifluoroacetate salt) was obtained from Bachem (UK) Ltd., St. Helens, Merseyside, U.K. 4-Cl-IAA was synthesized as described previously (*23*). 3-Methylene-2-oxindole and analogues were synthesized using the Peterson olefination procedure (*34*); full details and methods for the synthesis of 5-Me-IAA, 5-Cl-IAA, and 6-Cl-IAA are described elsewhere (*35*).

Reactivity of Methylene Oxindole and Analogues with Thiols. Spectral changes were examined using a Hewlett-Packard HP8452A spectrophotometer and a tandem cell (Hellma type 238, with two chambers each with 4.375 mm optical path length permitting spectra to be obtained before and after mixing). For kinetic measurements, ethanolic stock solutions of MOI were diluted in phosphate buffer (50 mM, pH 7.4) to give MOI concentrations of 0.5−1 mM (ethanol 2.5−10% v/v), and these were mixed in a Hi-Tech SF-61 stopped-flow spectrophotometer with equal volumes of freshly prepared GSH or CysSH (5−10 mM, pH adjusted to 7.4 using NaOH). Similar experiments involving MOI and the peptide Lys-Cys-Thr-Cys-Cys-Ala involved mixing a solution of MOI (0.05 mM) with equal volumes of peptide (0.25−1 mM). Loss of MOI was followed at 370 nm; five experiments were averaged and fitted to first-order kinetics. Rate constants were calculated from the linear plots of first-order rate constants against thiol concentrations.

Cell Survival Experiments. Chinese hamster lung fibroblast V79 cells, obtained from the European Collection of Cell Cultures, Salisbury, U.K., were maintained in Eagle's modified medium (EMEM) supplemented with 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, and with spinner modified EMEM (SMEM) supplemented with 7.5% FCS. Cells were subcultured by trypsin (1 × 0.5% porcine trypsin, 0.2% EDTA) removal of the cells. For cytotoxicity experiments, V79 cells were allowed to attach to 6 cm Petri dishes from spinner culture for at least 1 h in a humidified 37 °C 5% CO_2/air incubator before administration of the drug. MOI stock solutions (0.5−1 mM) were made in 1−6% ethanol/phenol red free Hanks' balanced salt solution. MOI dilutions (2 mL, 0.25−10 μM) were added to the cells for 2 h, and then washed with 2 mL of Hanks' solution 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.

Formation of Methylene Oxindoles from Oxidation of Indole Acetic Acids by HRP. Indole stock solutions (1 mM) were freshly prepared in Hanks' solution with 1% v/v ethanol and protected from light. IAA or analogues (0.1 mM, 2 mL) were added to a 6 cm Petri dish with 1.2 μg/mL HRP and incubated at 37 °C using the same conditions as in the cell survival experiments. Samples were removed over 2 h for HPLC analysis using a Waters 2690 HPLC with a model 996 photodiode array detector. Products were eluted on a reversed-phase RPB column (100 × 3.2 mm) with ammonium acetate (25 mM, pH 5.1), with a gradient of 25−75% of 75% acetonitrile over 10 min. MOI formation was measured at 250 nm, calibrating with synthesized standards.

Results

Kinetics of Reaction of Methyleneoxindoles with Thiols. The absorption spectra of MOI and analogues were obtained before and after mixing with thiols. Figure 1 shows spectral changes on mixing 5-Me-MOI with GSH in a two-compartment or 'tandem' spectrophotometer cell; changes occurred immediately on mixing. This methylene oxindole exhibited an absorbance maximum at ∼250 nm with shoulders at ∼290 and 370 nm, and the absorbances decreased immediately on reaction with GSH, similar to the changes reported for MOI and GSH (*7*). The kinetics

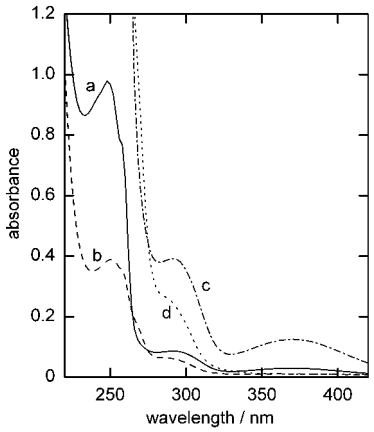

Figure 1. Absorption spectra of 5-methyl-3-methylene-2-oxindole and GSH (pH 7.4) in a tandem cell before and after mixing. 5-Me-MOI (0.05 mM) with GSH (0.5 mM): (a, —) before mixing; (b, ---) after mixing. 5-Me-MOI (0.25 mM) with GSH (2.5 mM): (c, — · —) before mixing; d (···) after mixing. (Concentrations are those in each chamber of the tandem optical cell, both with 4.375 mm optical path, before mixing.)

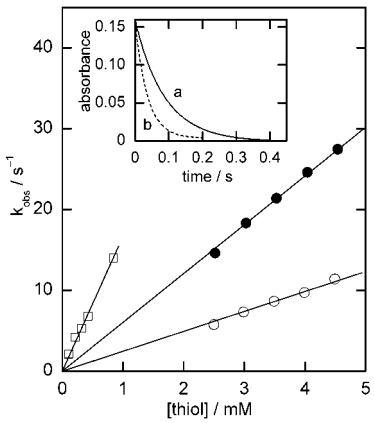

Figure 2. Dependence on thiol concentration of first-order rate constants for reaction of 3-methylene-2-oxindole (0.25 mM, or 0.05 mM for the peptide experiments) with excess GSH (○), cysteine (●), or the peptide Lys-Cys-Thr-Cys-Cys-Ala (□) at pH 7.4, 25 °C. Inset: absorbance (370 nm)/time profiles for MOI (0.25 mM) with 2.5 mM GSH (a, —) or cysteine (b, ···). Transients and data points are the means of 5 experiments; uncertainties are smaller than the symbol size.

of the reactions were measured by mixing MOI or analogues with at least a 10-fold excess of GSH or CysSH in milliseconds in a stopped-flow spectrophotometer, monitoring the spectral changes at 370 nm and fitting an exponential function to obtain first-order rate constants. Representative absorbance/time traces are shown in Figure 2, along with typical dependences of the first-order rate constants on thiol concentration. From the slopes of such plots, values of second-order rate constants for reaction between MOI or analogues with the thiols were estimated, shown in Table 1.

To compare the reactivities of free cysteine with cysteinyl residues in a peptide, the rate constant for reaction of MOI with the peptide Lys-Cys-Thr-Cys-Cys-Ala was measured similarly. Figure 2 includes the dependence on peptide concentration of the first-order rate constant characterizing the absorbance change, from which a second-order rate constant of $(1.67 \pm 0.04) \times 10^4$ M^{-1} s^{-1} was determined.

Addition of equine glutathione-*S*-transferase (0.59 unit/mL after mixing) did not accelerate the reaction between 5-F-MOI and GSH detectably.

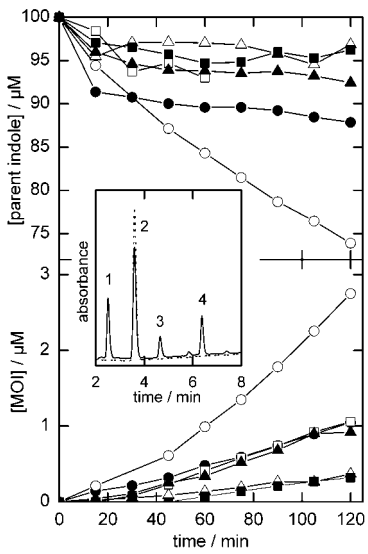

Figure 3. Loss of substituted indole-3-acetic acids (upper graph) or formation of substituted 3-methylene-2-oxindoles (lower graph) on reaction of indole-3-acetic acids (100 μM) with HRP (1.2 μg/mL) at 37 °C. Substituents: (○) none; (●) 5-methyl; (□) 5-F; (■) 4-Cl; (△) 5-Cl; (▲) 6-Cl. Inset: HPLC chromatogram (absorbance at 250 nm) of IAA before (···) and after (—) treatment with HRP after 2 h. Peaks: 1, oxindole-3-carbinol; 2, IAA; 3, indole-3-carbinol; 4, MOI.

Table 1. Second-Order Rate Constants for Reactions of Substituted Methylene Oxindoles withThiols at pH 7.4, 25 °C

	$k/10^3$ M^{-1} s^{-1}	
substituent	GSH	CysSH
none	2.46 ± 0.03	6.03 ± 0.04
5-Me	2.07 ± 0.02	4.50 ± 0.07
5-F	9.03 ± 0.08	14.4 ± 0.1
4-Cl	8.44 ± 0.14	10.9 ± 0.1
5-Cl	12.4 ± 0.2	17.8 ± 0.2
6-Cl	5.95 ± 0.06	11.0 ± 0.1

Loss of Indole Acetic Acids and Formation of Methylene Oxindoles on Treatment with Horseradish Peroxidase. The various indole-3-acetic acid derivatives (100 μM) were incubated with HRP (1.2 μg/mL) for up to 2 h at 37 °C. Figure 3 shows loss of the indoles, together with the amounts of the corresponding methylene oxindole formed. A typical chromatogram is inset.

Cytotoxicity of Methylene Oxindoles toward Mammalian Cells in Vitro. Clonogenic survival of Chinese hamster V79 fibroblast-like cells was measured after exposure to varying concentrations of methylene oxindoles for 2 h in an incubator at 37 °C. Figure 4 shows survival curves for all the compounds studied.

Discussion

Reactivity of methylene oxindoles toward thiols is not unexpected. As an α,β-unsaturated ketone, Michael addition of the thiol to the exocyclic double bond should occur (*36*), presumably forming the thioether (Scheme 1, **9**). Reaction between MOI and GSH was reported many years ago (*7*), although rate constants have not been reported, nor have the effects of substituents upon reactivity.

In the cellular environment, the steady-state concentration of MOI or analogues will be much less than that of the thiol pool, the decay of MOI by thiol reactions will be exponential, and so the half-life of MOI can be

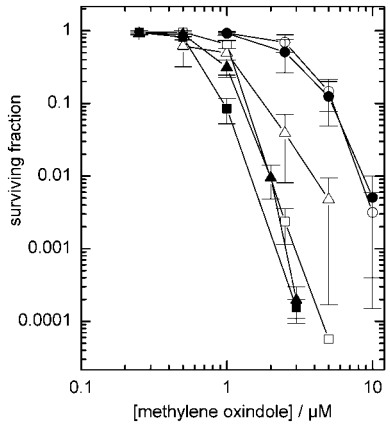

Figure 4. Clonogenic survival of V79 cells after exposure of varying concentrations of substituted 3-methylene-2-oxindoles for 2 h. Substituents: (○) none; (●) 5-methyl; (□) 5-F; (■) 4-Cl; (△) 5-Cl; (▲) 6-Cl. Means and standard errors of 3 independent experiments.

calculated as (ln 2)/(sum of products of rate constant × thiol concentration). Although CysSH is consistently more reactive toward MOI and analogues than GSH (Table 1), the latter is the dominant biological thiol, and except in the plasma [where the concentration of CysSH exceeds that of GSH (*37*)], the GSH concentration will provide an estimate of the maximum lifetime of MOI or analogues. Typical levels of intracellular nonprotein thiol (NPSH, principally GSH) are of the order of 5 mM, although values of around 10 mM have been reported (*38*). In the presence of ∼5 mM GSH at 25 °C, pH 7.4, the half-life of MOI is calculated as ∼60 ms, a value reduced by a factor of ∼4 or ∼5 on substitution with, e.g., F or Cl, respectively, at the 5-position (Table 1).

The half-lives of MOI or analogues may be used to estimate the approximate diffusion distances of these reactive intermediates in tissue. The root-mean-square diffusion distance in three-dimensional space is $(6Dt)^{1/2}$ where D is the diffusion coefficient and t the time. The diffusion coefficient of a small molecule in water at 37 °C may be estimated from the empirical relationship: $D = 1.013 \times 10^{-8}\ M^{-0.46}\ m^2\ s^{-1}$ (*39*); for MOI or 5-F-MOI, the relationship yields $D \sim 9 \times 10^{-10}\ m^2\ s^{-1}$. However, the cytosol of mammalian cells is more viscous than water: relative viscosities of 1.1−1.2 have been suggested (*40*), but overall impaired translational mobility of a small molecule in the cytoplasm compared to water by a factor of ∼4 has been measured (*41*), consistent with other high values (*42*, *43*). If $D \sim 3 \times 10^{-10}\ m^2\ s^{-1}$ and $t \sim 60$ or ~ 15 ms (MOI or 5-F-MOI, respectively, reacting with 5 mM GSH), then the diffusion distance is ∼10 or ∼5 μm, respectively. Even though diffusion involves other barriers than the cytoplasm, these calculations suffice to show that methylene oxindoles will not diffuse much more than cellular dimensions in tissue.

The ∼1.3−2.5-fold higher reactivity of CysSH over GSH toward MOI or analogues probably reflects the higher fraction of CysSH present in the thiolate form at pH 7.4. Thiols generally react as nucleophiles via the thiolate form, and although the higher the pK_a for thiol dissociation, the better the conjugate base as a nucleophile, this is usually less important than the effect of pK_a on thiol ionization [since the Brønsted coefficient correlating log k with pK_a for reactions of thiolate is <1 (*36*)]. The microscopic ionization constant (pK_a for SH dissociation of $^+H_3N\cdots SH$) for cysteine is ∼0.4 lower than that of GSH (*44*), so that at pH 7.4, the fraction of the thiol present as thiolate is ∼2.5-fold higher for CysSH compared to GSH, about the ratio of reactivity shown in Table 1 for MOI. The rate constant for reaction between MOI and the peptide Lys-Cys-Thr-Cys-Cys-Ala was found to be over twice the rate constant for free cysteine (Table 1), but the peptide has three cysteinyl residues. Reactivity of thiol groups in proteins is thus expected not to be significantly lower than that of free thiols.

Methyl substitution decreased, and F or Cl substitution increased, the reactivity of MOI toward both GSH and CysSH. While insufficient analogues were studied to establish a Hammett-type relationship, the results were consistent with the electron-donating and -withdrawing properties of the substituents. It is noteworthy that 5-F-MOI was ∼4-fold more reactive toward GSH than MOI, whereas fluorine substitution resulted in a full order of magnitude *reduction* in reactivity toward the key intermediate in peroxidase catalysis, HRP Compound I (*17*). The latter result was as expected from the dependence of rate constants on the electronic properties of the substituents (*30*, *31*), which show a marked redox relationship, with electron-donating substituents increasing ease of oxidation by Compound I. It was expected that halogen substitution should result in lower rates of formation of oxidative breakdown products on treatment with HRP, including methylene oxindoles. This was observed (Figure 3). Although the rate of production of 5-Me-MOI from 5-Me-IAA was unexpectedly slower than that of MOI from IAA (Figure 3, lower plot), the initial rate of loss of 5-Me-IAA was, as expected, greater than that of IAA (upper plot). The turnover of IAA and derivatives on treatment with HRP in the absence of hydrogen peroxide (the usual cofactor for oxidations catalyzed by HRP) is a complex subject discussed elsewhere (*2*, *33*). The present results indicate the extent of oxidative breakdown cannot simply be predicted from rate constants with Compound I.

While our early work in the area of HRP-catalyzed effects of IAA derivatives had demonstrated well-behaved structure−activity relationships for the effects of substituents on both Compound I reactivity (*30*, *31*) and peroxidation of liposomes (*45*), later studies failed to demonstrate clear-cut substituent effects on the cytotoxicity of IAA/HRP (*14*, *15*). In fact, the markedly enhanced cytotoxicity of the combination of 5-F-IAA/HRP compared to IAA/HRP was quite unexpected (*17*). The results of the present study go some way toward explaining the relative cytotoxic effects of IAA derivatives. Figure 4 shows that the halogenated derivatives of MOI are all significantly more toxic than MOI itself, but that the 5-methyl analogue is similar in behavior to MOI. This is consistent with the thiol reactivity shown in Table 1, and suggests that the effects of substituents on the reactivity of methylene oxindoles toward nucleophiles are more important than the effects on oxidative breakdown. The cytotoxicities of MOI or 5-F-MOI were about 3 orders of magnitude greater than those of the parent IAA or 5-F-IAA in the absence of HRP (*17*).

Whether thiols are the key target of the IAA/HRP treatment is not answered by this study. The relative reactivity of, e.g., MOI and 5-F-MOI toward other nucleophiles may be similar to reaction with thiols. Binding of HRP-catalyzed degradation products of IAA to histones was shown many years ago (*8*), but we demonstrated effects of IAA/HRP on DNA plasmids (*15*). Adding GSH

to IAA solutions that had been irradiated to generate the radical cation to initiate oxidation (but added after all radical reactions had ceased) was cytoprotective (*16*), and depleting cells of GSH by treatment with buthionine sulfoximine enhanced cytotoxicity (*17*). There was ~60% loss of intracellular GSH after treatment of V79 cells with 50 μM IAA or 5-F-IAA (*17*). Even if thiol centers are not the critical target, they are likely to be important to the overall response to the combination of IAA and derivatives with HRP.

We have discussed elsewhere (*16*, *21*) the potential for the use of IAA and derivatives in cancer therapy involving targeted enzyme activation of prodrugs, including gene therapy (*23*, *46*). The present study underlines the conclusion that the rational choice of prodrugs must involve a quantitative understanding of the chemical reactivity of breakdown products as much as the details of the prodrug/enzyme interaction.

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References

(1) Bandurski, R. S., Cohen, J. D., Slovin, J. P., and Reinecke, D. M. (1995) Auxin biosynthesis and metabolism. In *Plant Hormones—Physiology, Biochemistry and Molecular Biology* (Davies, P. J., Ed.) 2nd ed., pp 39−65, Kluwer Academic Publishers, Dordrecht, The Netherlands.

(2) Dunford, H. B. (1999) *Heme Peroxidases*, Wiley-VCH, New York.

(3) Candeias, L. P., Folkes, L. K., Dennis, M. F., Patel, K. B., Everett, S. A., Stratford, M. R. L., and Wardman, P. (1994) Free-radical intermediates and stable products in the oxidation of indole-3-acetic acid. *J. Phys. Chem.* **98**, 10131−10137.

(4) Hinman, R. L., Bauman, C., and Lang, J. (1961) The conversion of indole-3-acetic acid to 3-methyleneoxindole in the presence of peroxidase. *Biochem. Biophys. Res. Commun.* **5**, 250−254.

(5) Fukuyama, T. T., and Moyed, H. S. (1964) Inhibition of cell growth by photooxidation products of indole-3-acetic acid. *J. Biol. Chem.* **239**, 2392−2397.

(6) Hinman, R. L., and Lang, J. (1965) Peroxidase-catalyzed oxidation of indole-3-acetic acid. *Biochemistry* **4**, 144−158.

(7) Still, C. C., Fukuyama, T. T., and Moyed, H. S. (1965) Inhibitory oxidation products of indole-3-acetic acid. *J. Biol. Chem.* **240**, 2612−2618.

(8) Demorest, D. M., and Stahmann, M. A. (1972) The binding of the peroxidase oxidation products of indole-3-acetic acid to histone. *Biochem. Biophys. Res. Commun.* **47**, 227−233.

(9) Ricard, J., and Job, D. (1974) Reaction mechanisms of indole-3-acetate degradation by peroxidases. A stopped-flow and low-temperature spectroscopic study. *Eur. J. Biochem.* **44**, 359−374.

(10) Bednar, T. W., Linsmaier-Bednar, E. M., and King, C. M. (1976) Peroxidase-H_2O_2 catalysed incorporation of auxin derivatives into sRNA. *Biochem. Biophys. Res. Commun.* **72**, 761−767.

(11) Abreu, S. L., and Lucas-Lenard, J. (1976) Inhibition of cellular and viral protein synthesis by 3-methyleneoxindole. *Antimicrob. Agents Chemother.* **11**, 521−527.

(12) Kornet, M. J., Thio, A. P., and Thorstenson, J. H. (1977) Synthesis and cytotoxic activity of latentiated derivatives of 3-methyleneoxindole. *J. Pharm. Sci.* **66**, 1022−1024.

(13) Appleton, M. L., Larson, D. N., Skiles, G. L., Nichols, W. K., and Yost, G. S. (1990) Toxicity of 3-methyleneoxindole, a proposed reactive intermediate in the metabolism of 3-methylindole. *Adv. Exp. Med. Biol.* **283**, 245−248.

(14) Folkes, L. K., Candeias, L. P., and Wardman, P. (1998) Toward targeted 'oxidation therapy' of cancer: peroxidase-catalysed cytotoxicity of indole-3-acetic acids. *Int. J. Radiat. Oncol. Biol. Phys.* **42**, 917−920.

(15) Folkes, L. K., Dennis, M. F., Stratford, M. R. L., Candeias, L. P., and Wardman, P. (1999) Peroxidase-catalyzed effects of indole-3-acetic acid and analogues on lipid membranes, DNA and mammalian cells in vitro. *Biochem. Pharmacol.* **57**, 375−382.

(16) Folkes, L. K., and Wardman, P. (2001) Oxidative activation of indole-3-acetic acids to cytotoxic species—a potential new role for plant auxins in cancer therapy. *Biochem. Pharmacol.* **61**, 129−136.

(17) Folkes, L. K., Greco, O., Dachs, G. U., Stratford, M. R. L., and Wardman, P. (2002) 5-Fluoroindole-3-acetic acid: a prodrug activated by a peroxidase with potential for use in targeted cancer therapy. *Biochem. Pharmacol.* **63**, 265−272.

(18) Bagshawe, K. D. (1989) Towards generating cytotoxic agents at cancer sites. *Br. J. Cancer* **60**, 275−281.

(19) Deonarain, M. P., Spooner, R. A., and Epenetos, A. A. (1995) Genetic delivery of enzymes for cancer therapy. *Gene Ther.* **2**, 235−244.

(20) Melton, R. G., and Knox, R. J., Eds. (1999) *Enzyme-Prodrug Strategies for Cancer Therapy*, Kluwer Academic/Plenum Publishers, New York.

(21) Wardman, P. (2002) Indole-3-acetic acids and horseradish peroxidase: a novel prodrug/enzyme combination for targeted cancer therapy. *Curr. Pharmaceut. Des.* **8**, 1363−1374.

(22) Greco, O., Dachs, G. U., Wardman, P., Folkes, L. K., and Chaplin, D. J. (1999) Development of an enzyme/prodrug combination for gene therapy. *Proc. Am. Assn. Cancer Res.* **40**, 478.

(23) Greco, O., Rossiter, S., Kanthou, C., Folkes, L. K., Wardman, P., Tozer, G. M., and Dachs, G. U. (2001) Horseradish peroxidase-mediated gene therapy: choice of prodrugs in oxic and anoxic tumor conditions. *Mol. Cancer Ther.* **1**, 151−160.

(24) Molls, M., and Vaupel, P., Eds. (1998) *Blood Perfusion and Microenvironment of Human Tumors. Implications for Clinical Radiobiology*, Springer, Berlin.

(25) Kobayashi, S., Sugioka, K., Nakano, M., Takyu, C., Yamagishi, A., and Inaba, H. (1980) Excitation of indole acetate in myeloperoxidase-H_2O_2 system: possible formation of indole acetate cation radical. *Biochem. Biophys. Res. Commun.* **93**, 967−973.

(26) Kanofsky, J. R. (1988) Singlet oxygen production from the peroxidase-catalysed oxidation of indole-3-acetic acid. *J. Biol. Chem.* **263**, 14171−14175.

(27) Savitsky, P. A., Gazaryan, I. G., Tishkov, V. I., Lagrimini, L. M., Ruzgas, T., and Gorton, L. (1999) Oxidation of indole-3-acetic acid by dioxygen catalysed by plant peroxidases: specificity for the enzyme structure. *Biochem. J.* **340**, 579−583.

(28) Jantschko, W., Furtmüller, P. G., Allegra, M., Livrea, M. A., Jakopitsch, C., Regelsberger, G., and Obinger, C. (2002) Redox intermediates of plant and mammalian peroxidases: a comparative transient-kinetic study of their reactivity toward indole derivatives. *Arch. Biochem. Biophys.* **398**, 12−22.

(29) Candeias, L. P., Folkes, L. K., and Wardman, P. (1995) Amplification of oxidative stress by decarboxylation: a new strategy in antitumour drug design. *Biochem. Soc. Trans.* **23**, 262S.

(30) Candeias, L. P., Folkes, L. K., Porssa, M., Parrick, J., and Wardman, P. (1996) Rates of reaction of indoleacetic acids with horseradish peroxidase compound I and their dependence on the redox potentials. *Biochemistry* **35**, 102−108.

(31) Candeias, L. P., Folkes, L. K., and Wardman, P. (1997) Factors controlling the substrate specificity of peroxidases: kinetic and thermodynamics of the reaction of horseradish peroxidase compound I with phenols and indole-3-acetic acids. *Biochemistry* **36**, 7081−7085.

(32) Job, D., and Dunford, H. B. (1976) Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I. *Eur. J. Biochem.* **66**, 607−614.

(33) Dunford, H. B. (1995) One-electron oxidations by peroxidases. *Xenobiotica* **25**, 725−733.

(34) Peterson, D. J. (1968) A carbonyl olefination reaction using silyl-substituted organometallic compounds. *J. Org. Chem.* **33**, 780−784.

(35) Rossiter, S. (2002) A convenient synthesis of 3-methyleneoxindoles: cytotoxic metabolites of indole-3-acetic acids. *Tetrahedron Lett.* (in press).

(36) Douglas, K. T. (1988) Reactivity of glutathione in model systems for glutathione *S*-transferases and related enzymes. In *Glutathione Conjugation. Mechanisms and Biological Significance* (Sies, H., and Ketterer, B., Eds.) pp 1−41, Academic Press, London.

(37) Wardman, P., Folkes, L. K., Bentzen, S. M., Stratford, M. R. L., Hoskin, P. J., Phillips, H., and Jackson, S. (2001) Influence of plasma glutathione levels on radiation mucositis. *Int. J. Radiat. Oncol. Biol. Phys.* **51**, 459−463.

(38) Wahllãnder, A., Soboll, S., and Sies, H. (1979) Hepatic mitochondrial and cytosolic glutahione content and the subcellular distribution of GSH-*S*-transferases. *FEBS Lett.* **97**, 138−140.

(39) Fournier, R. L. (1999) *Basic Transport Phenomena in Biomedical Engineering*, Taylor & Francis, Philadelphia.

(40) Srivastava, A., and Krishnamoorthy, G. (1997) Cell type and spatial location dependence of cytoplasmic viscosity measured by time-resolved fluorescence microscopy. *Arch. Biochem. Biophys.* **340**, 159−167.

(41) Kao, H. P., Abney, J. R., and Verkman, A. S. (1993) Determinants of the translational mobility of a small solute in cell cytoplasm. *J. Cell Biol.* **120**, 175−184.

(42) Lang, I., Scholz, M., and Peters, R. (1986) Molecular mobility and nucleocytoplasmic flux in hepatoma cells. *J. Cell Biol.* **102**, 1183−1190.

(43) Seksek, O., Biwersi, J., and Verkman, A. S. (1997) Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. *J. Cell Biol.* **138**, 131−142.

(44) Rabenstein, D. L. (1973) Nuclear magnetic resonance studies of the acid−base chemistry of amino acids and peptides. I. Microscopic ionization constants of glutathione and methylmercury-complexed glutathione. *J. Am. Chem. Soc.* **95**, 2797−2803.

(45) Candeias, L. P., Folkes, L. K., Porssa, M., Parrick, J., and Wardman, P. (1995) Enhancement of lipid peroxidation by indole-3-acetic acid and derivatives: substituent effects. *Free Radical Res.* **23**, 403−418.

(46) Greco, O., Folkes, L. K., Wardman, P., Tozer, G. M., and Dachs, G. U. (2000) Development of a novel enzyme/prodrug combination for gene therapy of cancer: horseradish peroxidase/indole-3-acetic acid. *Cancer Gene Ther.* **7**, 1414−1420.

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