Halogenated indole-3-acetic acids as oxidatively activated prodrugs: new leads for targeted cancer therapy

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Abstract

Substituted indole-3-acetic acid (IAA) derivatives, plant auxins with potential for use as prodrugs in enzyme-prodrug directed cancer therapies, were oxidised with horseradish peroxidase (HRP) and toxicity against V79 Chinese hamster lung fibroblasts was determined. Rate constants for oxidation by HRP compound I were also measured. Halogenated IAAs were found to be the most cytotoxic, with typical surviving fractions of $<10^{-3}$ after incubation for 2 hours with 100 μM prodrug and HRP.

Introduction

The oxidation of the plant auxin indole-3-acetic acid (IAA) **1** (Figure 1) with horseradish peroxidase (HRP) has been extensively studied, and the mechanism is known to be complex.[1-5] The products have been shown to be inhibitory towards bacterial growth[6] and it has recently been shown that HRP oxidation of IAA produces a mammalian cell cytotoxin which causes damage to plasmid DNA.[7, 8] The low toxicity of IAA and the fact that it is not readily oxidised by endogenous mammalian peroxidases make it a suitable candidate for use in an enzyme-directed anticancer prodrug therapy.[9]

A previous study of some commercially available substituted IAA derivatives showed that, although it was possible to predict the relative rates of oxidation by HRP, there was no correlation between rate of oxidation and cytotoxicity.[10] Recently it was found that 5-fluoroindole-3-acetic acid, although a very poor substrate for HRP and slow to oxidise, gave very high cytotoxicity upon activation.[11,12] A number of other substituted indole-3-acetic acids were then synthesised in order to investigate further this unexpected result and develop a lead prodrug for *in vivo* studies, with the working

hypothesis that prodrugs substituted with halogens or other electron-withdrawing groups would exhibit improved cytotoxicity compared to IAA itself.

Methods

Substituted IAAs were synthesised using standard literature procedures or adaptations of published methodologies. 1-Methyl-, 5-fluoro-, 5-bromo-, and 5-benzyloxy-indole-3-acetic acids were purchased from Sigma-Aldrich, Poole, UK.

A number of indole-3-acetic acids with mono- or disubstitution in positions 4 - 7 (**2a-h**) were synthesised *via* a Fischer cyclisation (Scheme 1).[13, 14, 15]

Other substituted IAAs, which could not be unambiguously synthesised or gave poor yields by the Fischer method, were obtained by the direct 3-alkylation of the appropriate substituted indole. 7-Chloroindole was purchased from Lancaster Synthesis, Lancaster, UK, 5-iodoindole was purchased from Avocado, Port Heynsham, UK. Other haloindoles were synthesised by the Leimgruber-Batcho method (Scheme 3).[16] Arylindoles were obtained by a Suzuki coupling of 5-bromoindole.[17] The introduction of the acetic acid side chain was carried out *via* alkylation of the zinc salt followed by saponification of the ester to give the substituted IAAs **3a** – **3h** (Scheme 2).[18] *N*-Methylated compounds **4a** and **4b** were also prepared using sodium hydride / iodomethane (Scheme 3).

Each IAA derivative was oxidised with HRP, and the cytotoxicity against Chinese hamster lung fibroblasts (V79 cells) was determined by a clonogenic assay as previously described.[8] Cells were treated with 2 mL indoles (50 µM or 100 µM) and HRP (1.2 µg/mL) for 0-2 h, then washed and left to form colonies 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.

The measurement of the rate of reaction of each IAA with HRP compound I, formed from a one second premixing of equimolar HRP and hydrogen peroxide (0.47 - 0.76 μM), was carried out as previously described, at pH 7, using double-mix stopped-flow spectrophotometry, monitoring the formation of HRP compound II at 411 nm.[8, 10, 19] Five experiments were averaged and fitted to first order kinetics. Rate constants were calculated from the linear plots of first-order rate constants against five IAA concentrations.

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Results and Discussion

Results are shown in Table 1, with the results for the previously studied IAA derivatives given as a comparison.[11,19] No toxicity was seen when cells were incubated with either HRP or 100μM prodrug alone.

Rates of oxidation are broadly in line with mechanistic predictions, i.e. the presence of electron-donating groups enhances the rate of oxidation by HRP compound I. Oxidation rates show some variation with substituent position. There is no direct correlation between rate of oxidation and subsequent toxicity, although many of the monosubstituted IAAs that are cytotoxic have an oxidation rate constant in the range $5 \times 10^2 - 5 \times 10^3$ $M^{-1}s^{-1}$. Disubstituted halogenated IAAs were generally not toxic after oxidation. The results seem to indicate that both steric and electronic effects may be involved, as prodrugs with similar oxidation rates show a wide variation in subsequent cytotoxicity. For example, it is interesting to note that 4-chloro-IAA **3c**

and 5-chloro-IAA **2c** have equal rates of oxidation, but oxidation of **3c** results in no observable toxicity whereas the products of oxidation of **2c** give a cell surviving fraction of 3×10^{-3}. Prodrugs that are very slowly oxidised ($k < 1 \times 10^3$ M^{-1} s^{-1}) gave little cytotoxicity, with the notable exception of 5-fluoro-IAA. In these cases, the slow turnover of the prodrug by HRP may limit any cytotoxic effect of the products generated.

6-Chloro IAA **3f** exhibited the highest toxicity upon HRP activation, with further experiments using 50 μM prodrug plus HRP giving a measured cell surviving fraction after 2 hours incubation of 8.5×10^{-4}. This analogue is now the lead prodrug for further studies of *in vitro* and *in vivo* drug targeting strategies (Figure 2). Such strategies include antibody, gene, or polymer targeting of HRP to tumours (antibody / gene / polymer directed enzyme-prodrug therapy; ADEPT, GDEPT, PDEPT) for selective activation of the prodrug at the tumour site.[11,20,21]

Preliminary studies suggest that 3-methyleneoxindole **5** (Figure 3) is an important toxic oxidation product in this system. It is susceptible to attack by nucleophiles, and therefore could react with cellular thiols and also has the potential to be DNA-binding.[11] The presence of electron-withdrawing groups in substituted 3-methyleneoxindoles would be expected to enhance the toxicity, consistent with the general trend observed for substituted IAAs, We are currently investigating the synthesis and reactivity of **5** and substituted analogues, and have demonstrated that these compounds exhibit toxicities broadly consistent with those of the corresponding oxidised prodrugs.[22,23] The toxicity of the most rapidly oxidised 2-methyl-substituted IAA analogues cannot be explained in terms of an oxindole product, and so it is expected that there is at least one other mechanism of toxicity involved, which requires further investigation.

Conclusion

Halogenated indole-3-acetic acids, in particular 6-chloro IAA, have been identified as potent lead prodrugs for peroxidase-mediated targeted cancer therapy. The parent prodrugs exhibit negligible toxicity at 100μM, and so efficient targeting of the activating enzyme has the potential to give a very favourable therapeutic ratio for this enzyme-prodrug system.

Acknowledgements

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Captions:

Table 1: Oxidation rates of IAA derivatives and cytotoxicity of products

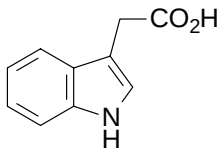
Figure 2: Toxicity of 6-chloroindole-3-acetic acid / HRP combination to V79 cells

Rossiter Table 1

reference	substituent	Rate constant with HRP cmp I x 10^3 $M^{-1}s^{-1}$(± s.e.m)	Surviving fraction after 2h incubation, mean (± s.e.m), n = 3[a]
2a	5-CF_3O	0.3 ± 0.01	1
	5-F	0.4 ± 0.01[11]	< 1 x 10^{-5}
4a	1-Me, 5-F	0.4 ± 0.03	1
2b	4-Cl, 6-Cl	0.6 ± 0.02	1
4b	1-Me, 6-Cl	0.9 ± 0.1	1
3a	7-Cl	0.9 ± 0.1	4 x 10^{-5} [b]
3b	4-F	1.0 ± 0.06	9 x 10^{-5} (± 7 x 10^{-5})
3c	4-Cl	1.4 ± 0.01	1
2c	5-Cl	1.4 ± 0.04	3 x 10^{-3} (± 2 x 10^{-3})
	5-Br	1.7 ± 0.04	4 x 10^{-5} (± 3 x 10^{-5})
3d	6-F	1.8 ± 0.02	1 x 10^{-2} (± 7 x 10^{-3}) [c]
	1-Me	3.7 ± 0.03	1 x 10^{-2} (± 5 x 10^{-3})
	none	3.8 ± 0.1[19]	1 x 10^{-2} (± 5 x 10^{-3})
3e	5-I	3.9 ± 0.2	1
2d	5-Me, 7-F	4.1 ± 0.9	1
2e	5-Cl, 7-F	5.3 ± 0.4	1
2f	4-Me, 7-F	5.8 ± 0.3	1
3f	6-Cl	7.5 ± 0.2	< 1 x 10^{-5}
	5-BnO	21 ± 0.03	0.2 (± 0.1)
	5-MeO	22 ± 0.1[19]	1
2g	5-Me	41 ± 2	1
3g	5-Ph	46 ± 3	1
2h	2-Me, 5-F	88 ± 3	1
3h	5-(4-ClC_6H_4)	103 ± 0.2	1
	2-Me	435 ± 6[19]	4 x 10^{-3} (± 2 x 10^{-3})
	2-Me, 5-MeO	1710 ± 130[19]	4 x 10^{-2} (± 1 x 10^{-2})

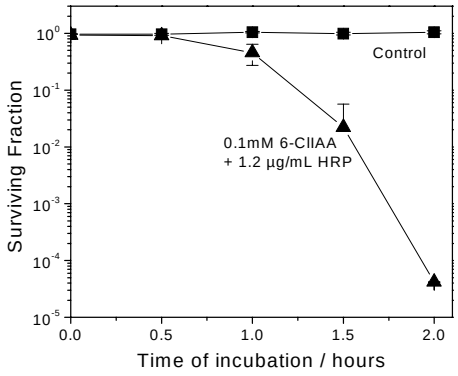
a: 100 μM prodrug + 1.2 μg/mL HRP.
b: n = 1
c: n = 2

Rossiter Figure 1

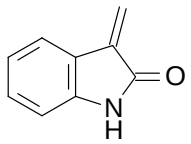


1

Rossiter Figure 2



Rossiter Figure 3:



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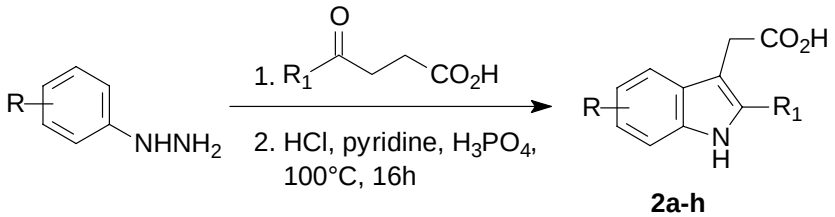
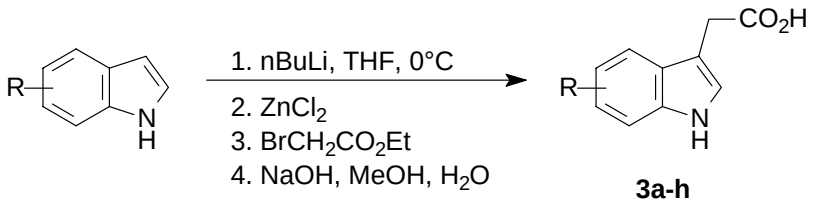
Rossiter Scheme 1



2a-h

2a: R = 5-CF_3O; R_1 = H
2b: R = 4-Cl, 6-Cl; R_1 = H
2c: R = 5-Cl; R_1 = H
2d: R = 5-Me, 7-F; R_1 = H
2e: R = 5-Cl, 7-F; R_1 = H
2f: R = 4-Me. 7-F; R_1 = H
2g: R = 5-Me; R_1 = H
2h: R = 5-F; R_1 = Me

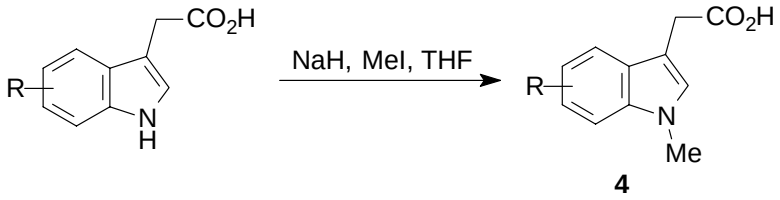
Rossiter Scheme 2



3a-h

3a: 7-Cl
3b: 4-F
3c: 4-Cl
3d: 6-F
3e: 5-I
3f: 6-Cl
3g: 5-Ph
3h: 5-(4-ClC$_6$H$_4$)

Rossiter Scheme 3



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4a: R = 5-F
4b: R = 6-Cl