Horseradish Peroxidase-mediated Gene Therapy: Choice of Prodrugs in Oxic and Anoxic Tumor Conditions[1]

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Abstract

We have previously proposed the plant enzyme horseradish peroxidase (HRP) and the plant hormone indole-3-acetic acid (IAA) as an enzyme/prodrug combination for cancer gene therapy. In the current study, we evaluated the potential of HRP/IAA for gene-directed enzyme/prodrug therapy in three human tumor cell lines (T24 bladder carcinoma, MCF-7 breast adenocarcinoma, and FaDu nasopharyngeal squamous carcinoma) and one endothelial cell line (HMEC-1). The action of 10 IAA analogues in combination with HRP was studied *in vitro* in normoxic conditions as well as in the extreme tumor conditions of anoxia. Compounds characterized by prompt normoxic or anoxic cytotoxic activation and high HRP transfectant killing or selectivity were identified. Some variations were observed in the response of cells of different origin, with IAA, 1-Me-IAA, and 5-Br-IAA representing the most promising candidates for HRP gene therapy. In particular, 5-Br-IAA showed a very prompt and selective activation in anoxia. A strong bystander effect was produced by activated IAA and analogues because 70–90% cell kill was obtained when only 5% of the cells expressed the HRP enzyme. These results indicate that HRP/IAA represents an effective system for enzyme/prodrug-based anticancer approaches, and further improvements could be achieved by the use of novel IAA derivatives.

Introduction

One of the major goals of antitumor therapies is to target toxic agents to tumor cells selectively and specifically while sparing normal tissue from damage. This may be achieved by gene therapy that can combine highly specific gene delivery and gene expression. To date, more than 400 gene therapy clinical trials have been undertaken worldwide, more than half of which relate to cancer (1).

A promising approach in the design of therapeutic genes for cancer gene therapy is suicide gene therapy or GDEPT.[3] GDEPT is a two-step strategy: initially, a foreign gene encoding a nontoxic enzyme is delivered to the tumor. In a second step, a prodrug is administered, which is converted into a potent cytotoxin by the enzyme expressed at the target (reviewed in Ref. 2).

In the choice of the appropriate combination for GDEPT, a number of properties should be considered. The enzyme should have high catalytic activity under physiological conditions and fast and efficient prodrug activation even at low concentrations of the substrate (high K_{cat} and low K_m), without dependence on further catalysis by other cellular enzymes. The induced cytotoxicity should be cell cycle phase or proliferation independent to kill a wide range of tumor cell populations. The toxic agent should also have a half-life that allows transport to the surrounding untransfected cells (bystander effect) but ensures that any drug escaping into the circulation will be inactive. The bystander phenomenon, initially described by Moolten (3), can be defined as an extension of the killing effects of the active drug to untransfected neighboring cells. This implies that even if only a fraction of the target cells are genetically modified and express the therapeutic gene, tumor eradication may still be achieved.

We have developed a novel GDEPT system consisting of HRP and the nontoxic plant hormone IAA (4). Using this system, we demonstrated fast and efficient *in vitro* prodrug activation in a preliminary report (4). When compared with the well-established herpes simplex virus-1 thymidine kinase/ganciclovir combination, HRP/IAA showed increased *in vitro* toxicity in normoxia and, more significantly, in the extreme tumor conditions of anoxia. This could imply a therapeutic advantage because hypoxia is common to solid tumors and presents an adverse prognostic indicator (reviewed in Ref. 5). The HRP/IAA system has the potential to be used in a variety of anticancer strategies (6). Besides GDEPT, specific HRP targeting to the tumor could be achieved with HRP-conjugated antibodies [antibody-directed enzyme/prodrug therapy (7)] or polymers [polymer-directed enzyme/prodrug therapy (8)]. IAA is well tolerated in humans (9), and nonspecific activation in normal tissue is unlikely to take place because mammalian peroxidases failed to convert it into a cytotoxin at therapeutically significant prodrug doses (10–12).

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[3] The abbreviations used are: GDEPT, gene-directed enzyme/prodrug therapy; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; CMV, cytomegalovirus; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; SI, selectivity index.

In the present work, we evaluate the potential of HRP/IAA for GDEPT in three human tumor cell lines and one endothelial cell line. The action of 10 IAA derivatives in combination with HRP was studied in normoxic and anoxic conditions. The data reported indicate that at least two analogues besides IAA induce efficient toxicity and bystander killing, suggesting the efficacy and selectivity of the HRP/IAA system for enzyme/prodrug-based anticancer approaches.

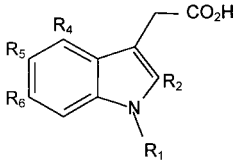
Materials and Methods

Cell Culture. T24 bladder carcinoma and MCF-7 mammary adenocarcinoma (both from the European Collection of Cell Cultures, Salisbury, United Kingdom), FaDu nasopharyngeal squamous carcinoma (American Type Culture Collection, Manassas, VA), and HMEC-1 dermal microvascular endothelial (13) cell lines were maintained in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co. Aldrich, Gillingham, United Kingdom), and 2 mM L-glutamine (Life Technologies, Inc.) in a humidified incubator at 37°C and 5% CO_2/air. Only cells that tested negative for *Mycoplasma* infection were used.

Hypoxic Conditions. For experiments in anoxic and hypoxic conditions, cells were incubated at 37°C in an anaerobic glove cabinet (DON Whitley Scientific Limited, Shipley, United Kingdom) with 5% CO_2, 5% H_2, 90% N_2, and palladium catalyst (anoxia) or in air-tight Perspex boxes flushed continuously with a humidified gas mixture containing 0.1% O_2, 5% CO_2, and 94.9% N_2 (hypoxia). For all anoxic/hypoxic experiments, cultures were manipulated in the anaerobic cabinet, and plastics and fluids were preincubated in the cabinet for 24–48 h before use to remove residual oxygen.

Plasmid DNA and Cell Transfection. The plasmid pRK34-HRP (14) was kindly provided by Dr. D. F. Cutler (University College London, London, United Kingdom); the control plasmid pCMV-CD4 was constructed as described previously (15). In both cases, gene expression was driven by the CMV early promoter. Transient transfectants were obtained by exposing the cells to complexes of DNA, Lipofectin (Life Technologies, Inc.), and integrin-targeted peptides (16) and assayed for gene expression after 24 h.

Detection of HRP Expression. Antibody staining was carried out by fixing cell suspensions in 3% paraformaldehyde (Sigma Chemical Co. Aldrich)/PBS for 20 min at room temperature. After centrifugation, the pellets were rinsed in PBS and incubated for 15 min at room temperature in 15 mM glycine (Sigma Chemical Co. Aldrich)/PBS. Nonspecific binding was blocked by the addition of wash buffer [5% FCS/1% Tween 20 (Sigma Chemical Co. Aldrich)/PBS]. The samples were centrifuged and incubated for 1 h at room temperature with rabbit polyclonal anti-HRP (Dako, Ely, United Kingdom) diluted 1:200 in 10% FCS/wash buffer. After extensive rinsing in wash buffer (at least three times), the samples were resuspended in TRITC-conjugated swine antirabbit immunoglobulins (Dako) diluted 1:200 in 10% FCS/wash buffer (1 h, room temperature). The cells were rinsed in wash buffer and resuspended in HBSS (Life Technologies, Inc.) for fluorescence-activated cell-sorting analysis on a Becton Dickinson FACScan. Cells were scored as positive if they showed an increase in fluorescence with respect to CD4-expressing cells.



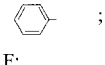
IAA	: $R_{1\text{-}6}$ = H		
1-Me-IAA	: R_1 = CH_3;	$R_{2\text{-}6}$	= H
2-Me-IAA	: R_2 = CH_3;	$R_{1,3,\text{-}6}$	= H
5-MeO-IAA	: R_5 = CH_3O;	$R_{1\text{-}4,6}$	= H
2-Me,5-MeO-IAA	: R_2 = CH_3;	R_5	= CH_3O; $R_{1,3,\text{-}4,6}$ = H
5-BnO-IAA	: R_5 = 	; $R_{1\text{-}4,6}$	= H
5-Ph-IAA	: R_5 = 	; $R_{1\text{-}4,6}$	= H
6-F-IAA	: R_6 = F;	$R_{1\text{-}5}$	= H
4-Cl-IAA	: R_4 = Cl;	$R_{1\text{-}3,5,\text{-}6}$	= H
5-F-IAA	: R_5 = F;	$R_{1\text{-}4,6}$	= H
5-Br-IAA	: R_5 = Br;	$R_{1\text{-}4,6}$	= H

Fig. 1. Prodrugs studied in combination with the enzyme HRP for gene therapy.

HRP activity was analyzed using a modified 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma Chemical Co. Aldrich) assay, as described previously (4). The total cellular protein content in the samples was determined by using a commercial protein assay kit (Bio-Rad, Hemel Hempstead, United Kingdom). The HRP activity was expressed as units of enzyme per microgram of total cellular protein. A unit is defined as the amount of enzyme that produces an increase of 1 unit of absorbance at 652 nm ($A_{652\ nm}$) per minute.

For Western blots, cell extracts were subjected to SDS-PAGE as described previously (4). Detection of immunoreactive bands was performed using the enhanced chemiluminescence technique (ECL kit; Amersham Pharmacia Biotech, Amersham, United Kingdom).

Compounds. The IAA analogues used are illustrated in Fig. 1. IAA, 1-Me-IAA, 2-Me-IAA, 5-MeO-IAA, 2-Me,5-MeO-IAA, 5-BnO-IAA, 5-F-IAA, and 5-Br-IAA were purchased from Sigma Chemical Co. Aldrich.

5-Ph-IAA, 6-F-IAA, and 4-Cl-IAA were prepared as follows, using adaptations of procedures described previously in the literature (17–19). A solution of the indole (2–10 mmol) in tetrahydrofuran was cooled to 0°C, and *n*-butyllithium (1.1 equivalents) was added. The yellow solution was stirred at 0°C for 20 min, zinc chloride (1.1 eq. of a 1 M solution in diethyl ether) was added, and the mixture was stirred for 2 h at room temperature. Ethyl bromoacetate (1.1 eq.) was added, and the mixture was stirred overnight. After the addition of water, the mixture was partitioned into ethyl acetate and 1 M HCl. The organic layer was dried ($MgSO_4$), the solvent was removed *in vacuo,* and the crude product was purified by flash column chromatography (3:1, hexane:ethyl acetate) to furnish the substituted ethyl indole-3-acetate. The ester was heated under reflux in a solution of NaOH in 1:1 methanol:water for 4 h. The indole was precipitated by acid-

ification (HCl) and purified by recrystallization from chloroform or methanol:water. All air- and water-sensitive reactions were carried out under nitrogen. Glassware was oven-dried and cooled in an anhydrous atmosphere before use. Products were analyzed for purity using a Waters Integrity high-performance liquid chromatography mass spectrometer system, nuclear magnetic resonance spectra were recorded on a Jeol MY60 spectrometer, and melting points (uncorrected) were measured using a Gallenkamp hot plate apparatus. Elemental analyses were carried out by Medac (Egham, United Kingdom) and were in agreement with calculated values.

Clonogenic Assay. Exponentially growing cells were plated at low density and exposed to IAA or derivatives for 2 or 24 h in phenol-red-free HBSS in a 5% CO_2 incubator at 37°C. For experiments under anoxia/hypoxia, cells were preplated in the anaerobic cabinet and, after incubation for 5–6 h in the cabinet or under 0.1% O_2 to ensure anoxic/hypoxic conditions, exposed to the prodrugs.

In conditioned medium-switch experiments, cells transfected with pRK34-HRP (HRP^+) were exposed to HBSS containing a range of IAA concentrations for 2 h. This medium was subsequently transferred to preplated cells transfected with the control plasmid pCMV-CD4 (HRP^-) for a subsequent 2-h incubation.

To measure the bystander effect, HRP^+ and HRP^- populations were mixed in different proportions and exposed to IAA for 24 h. After drug exposure, cells were counted using a hemocytometer and replated at low density for clonogenic survival.

After treatment, cells were rinsed with PBS and grown for 8–20 days in complete DMEM supplemented with feeder cells (V79 cells exposed to 250 Gy of ^{60}Co irradiation). After fixation and staining with 2.5% (w/v) crystal violet (Sigma Chemical Co. Aldrich) in isomethylated spirit, colonies of >50 cells were scored. Surviving fractions were evaluated relative to HBSS-treated controls. The concentration of prodrug required to reduce cell survival by 50% (IC_{50}) was estimated from the survival curves. At least three independent experiments were conducted (triplicate samples).

Growth Inhibition. Cells were preplated in 96-well plates (2000 cells/well, 8 wells/drug concentration) and exposed to the prodrugs as described in the previous section. After drug exposure, the cells were grown until control plates reached confluence (3–5 days) and assayed using the CellTiter 96 AQ_{ueous} Assay (Promega, Southampton, United Kingdom) according to manufacturer's instructions. Briefly, the culture medium was supplemented with MTS and phenazine methosulfate at final concentrations of 166 μg/ml and 12.5 μM, respectively, and left to react for 2 h. The plates were agitated to ensure complete mixing and scanned on a multiplate reader (Labsystems Multiskan MCC/340) at 492 nm. Cell density was evaluated relative to HBSS-treated controls. The IC_{50} was estimated from the curves.

Results

HRP/IAA Gene Therapy of Human Cells. To demonstrate wide applicability of HRP-mediated GDEPT, four cell lines of human origin (T24 bladder carcinoma, MCF-7 breast adenocarcinoma, FaDu nasopharyngeal squamous carcinoma, and microvascular endothelial cell line HMEC-1) were evaluated. Cells were transiently transfected with the pRK34-HRP construct, in which the HRP cDNA was previously fused to the signal sequence from the human growth hormone and the KDEL retention motif (14). The KDEL tag causes accumulation of the HRP in the endoplasmic reticulum and the nuclear envelope (4, 14), preventing secretion of the enzyme. This would be an advantage *in vivo,* where nonspecific prodrug activation at sites distant from the tumor is undesirable. As assessed by immunostaining, the transfection efficiencies were 20–25% in T24 cells, 16–20% in MCF-7 cells, 10–14% in FaDu cells, and 18–20% in HMEC-1 cells. As expected, untransfected cells and mock-transfected (HRP^-) cells expressing the marker CD4 did not stain positive for HRP (results not shown). Synthesis of an immunoreactive M_r 52,000 protein and competent peroxidase activity were confirmed in all HRP^+ cells by Western blotting (results not shown) and HRP assay. In T24, MCF-7, and FaDu cells, HRP activity was 3.6 ± 0.9, 1.1 ± 0.6, and $0.8 \pm 0.2 \times 10^{-5}$ units/μg total protein, respectively. Compared with the other cell lines, a higher peroxidase activity was detected in HMEC-1 cells ($17 \pm 3 \times 10^{-5}$ units/μg total protein), which was consistent with an increased number of HRP-containing plasmids/cell, compared with the other cell lines. No detectable HRP protein production or catalyst activity could be measured in HRP^- cells (data not shown).

Susceptibility of HRP-expressing T24, MCF-7, and FaDu cells to prodrug treatment was assessed by exposing the cells to increasing concentrations of IAA (Fig. 2). Results on T24 cells confirmed findings reported previously (4). After only a 2-h exposure, significant cell killing was induced in HRP transfectants, whereas no toxicity was observed in HRP^- cells (Fig. 2*A*). However, above 4 mM, a rapid and dramatic decrease in survival was observed both in the HRP^- and HRP^+ populations. This effect was due to a significant acidification of the IAA-containing medium because when the pH was adjusted to 7.4, no toxicity was detected in HRP^- cells exposed for 2 h to up to 20 mM IAA (results not shown). Doses above 4 mM were therefore not included in the survival curves.

A higher efficacy of the HRP/IAA combination was observed after prolonged (24 h) incubation (Fig. 2*B*). In all of the cell lines studied, expression of the HRP enzyme significantly enhanced sensitivity to IAA, with levels of cell kill of up to 4–5 logs. Some toxicity was also observed in HRP^- cells at concentrations $\geq$ 1 mM. Equivalent doses that reduced the surviving fraction to 50% (IC_{50}s) for HRP^+ cells were compared with the corresponding HRP^- populations. The ratios of IC_{50} for HRP^- cells:IC_{50} for HRP^+ cells were defined as SI. After a 24-h incubation, SIs of 36, 55, and 85 were measured in T24, MCF-7, and FaDu cells, respectively. After analysis of the full survival curves, the response of these three tumor cell lines to HRP/IAA was not found to differ considerably at doses of prodrug below 1 mM (Fig. 2*B*), with MCF-7 cells being slightly more sensitive. At higher concentrations of IAA, HRP^+ T24 cells showed an increase in cell kill of 1–2 logs compared with HRP^+ FaDu and MCF-7 cells.

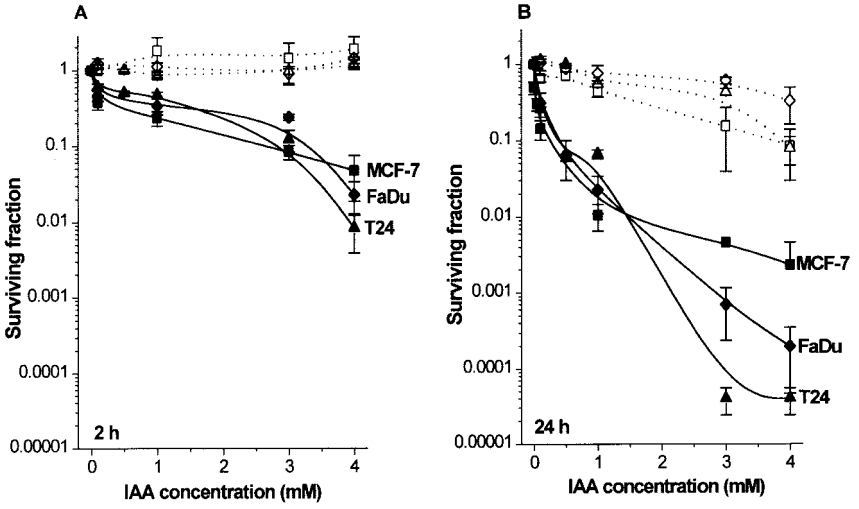


Fig. 2. HRP/IAA GDEPT. Clonogenic survival of HRP$^-$ and HRP$^+$ tumor cells exposed for 2 h (*A*) or 24 h (*B*) to increasing concentrations of the prodrug IAA. △, HRP$^-$ T24 cells; ▲, HRP$^+$ T24 cells; □, HRP$^-$ MCF-7 cells; ■, HRP$^+$ MCF-7 cells; ◇, HRP$^-$ FaDu cells; ◆, HRP$^+$ FaDu cells. Means and SEs of at least three independent experiments are indicated. The lines are interpolated.

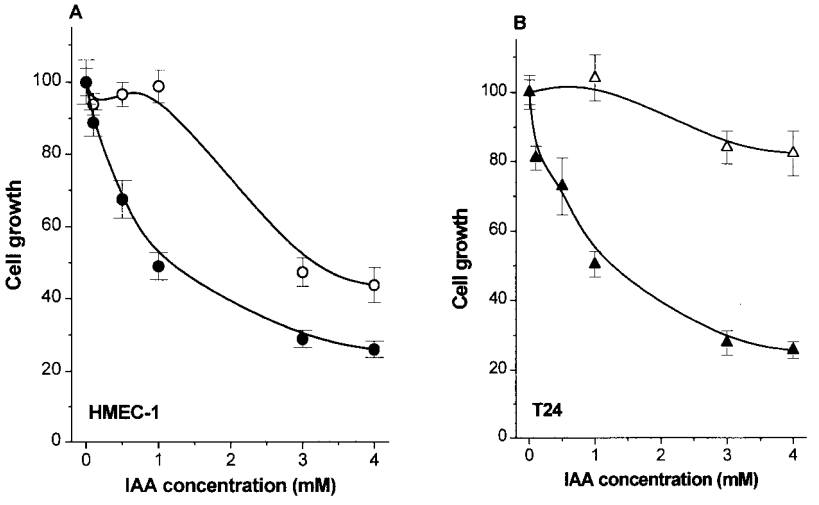


Fig. 3. Growth inhibition (MTS assay) of HRP$^-$ and HRP$^+$ human microvascular endothelial HMEC-1 (*A*) and T24 bladder carcinoma (*B*) cells in the presence of IAA. ○, HRP$^-$ HMEC-1 cells; ●, HRP$^+$ HMEC-1 cells; △, HRP$^-$ T24 cells; ▲, HRP$^+$ T24 cells. The means of three independent experiments ± SE are shown. The lines are interpolated.

The potential of the HRP/IAA combination to target the tumor vasculature was evaluated in the HMEC-1 endothelial cell line. The growth of HMEC-1 cells was monitored using the MTS assay (Fig. 3*A*) because these cells do not form discrete colonies. For comparative purposes, the growth of T24 cells was analyzed under the same experimental conditions (Fig. 3*B*). After 24 h of IAA treatment, inhibition of proliferation was detected in HRP$^+$ HMEC-1 cells, following a dose response very similar to that of T24 HRP transfectants (Fig. 3). However, compared with HRP$^-$ T24 cells, HRP$^-$ HMEC-1 cells appeared to be more sensitive to IAA, resulting in a decrease in selectivity (HMEC-1 cells, SI = 3; T24 cells, SI > 4, as assessed using the MTS assay).

Use of IAA Derivatives for GDEPT. To increase the antitumor potential of the HRP/IAA system, a panel of IAA derivatives (Fig. 1) were studied in combination with HRP.

5-MeO-IAA, 6-F-IAA, and 4-Cl-IAA showed effects very similar to IAA in normoxia (Fig. 4), but almost no selective cytotoxicity could be detected in HRP$^+$ cells in anoxia (Fig. 5). 2-Me,5MeO-IAA induced nearly no toxicity in both HRP$^-$ and HRP$^+$ cells under any of the exposure conditions analyzed (Figs. 4 and 5). 5-BnO-IAA and 5-Ph-IAA showed fast and effective HRP$^+$ cell killing in both normoxia and anoxia, but they also induced high nonspecific toxicity in HRP$^-$ control cells (Figs. 4 and 5).

The action of the compounds characterized by prompt normoxic (1-Me-IAA and 2-Me-IAA) as well as anoxic (in particular, 5-Br-IAA) cytotoxic activation and high HRP$^+$ cell kill and selectivity (1-Me-IAA, 5-Br-IAA, and 5-F-IAA) was further investigated in detailed survival experiments using different cell types. T24, MCF-7, and FaDu cells were exposed for 2 or 24 h to the analogues 1-Me-IAA, 2-Me-IAA, 5-F-IAA, and 5-Br-IAA in normoxia.

After prolonged incubation with the cellular monolayers (24 h), the prodrugs studied induced high and selective sensitization in HRP$^+$ cells in all three tumor lines (Fig. 6) and in HMEC-1 cells (data not shown). In particular, 1-Me-IAA showed the highest SIs (SI = 740 in T24, 71 in MCF-7, and 50 in FaDu cells) and HRP$^+$ cell kill, especially at low doses (Fig. 6*A*). 2-Me-IAA, on the other hand, induced surviving

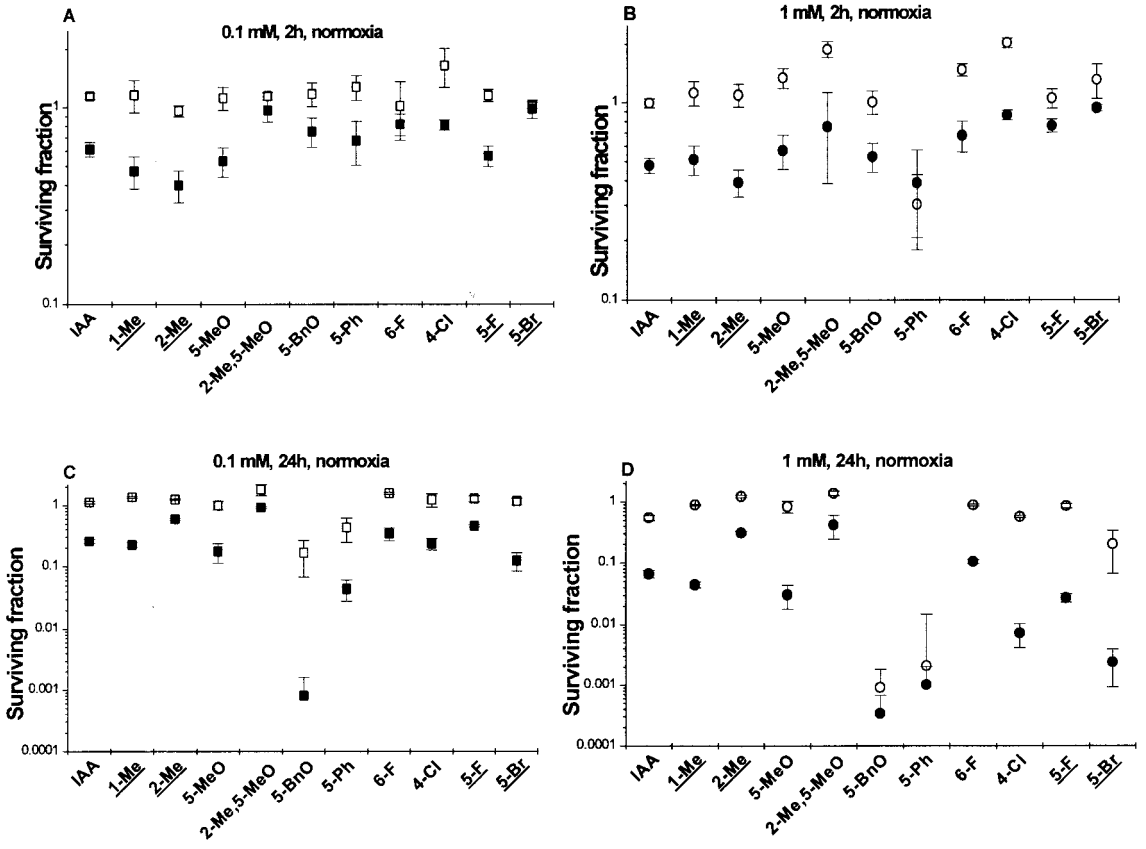


Fig. 4. Use of IAA derivatives for GDEPT in normoxia. T24 cells were exposed to a panel of IAA analogues (Fig. 1) at the concentrations of 0.1 mM (*A* and *C*) or 1 mM (*B* and *D*) for 2 h (*A* and *B*) or 24 h (*C* and *D*). □, HRP^- cells, 0.1 mM prodrug; ■, HRP^+ cells, 0.1 mM prodrug; ○, HRP^- cells, 1 mM prodrug; ●, HRP^+ cells, 1 mM prodrug. The means of three independent clonogenic assay experiments ± SE are indicated. Prodrugs adopted in subsequent survival studies are *underlined*.

fractions of less than 10% only at doses above 1 mM (Fig. 6*B*). 5-F-IAA, although very effective in T24 (SI = 88) and FaDu (SI = 60) cells, was characterized by nonspecific toxicity in HRP^- MCF-7 cells, resulting in a SI of only 1.6 in this cell line (Fig. 6*C*). Finally, 5-Br-IAA was efficient and selective in all of the cell lines analyzed (SIs were between 71 and 100; Fig. 6*D*) In this case, prodrug levels of up to 1 mM were studied because of toxicity in HRP^- cells.

The above-mentioned IAA derivatives were also tested in combination with HRP in T24 cells in the extreme tumor conditions of anoxia (Fig. 7). Compared with normoxic exposure, the effects of IAA, 1-Me-IAA, 2-Me-IAA, and 5-F-IAA were not modified after either a 2-h (data not shown) or a 24-h incubation (Figs. 2*B*, 5, and 7, *A*–*D*). Similar findings were obtained after hypoxic (0.1% O_2) IAA treatment (data not shown). Unexpectedly, 5-Br-IAA showed toxicity in anoxia at 2 h, with 2–3 log cell kill induced at 3 mM (Fig. 7*E*), whereas normoxic 2-h treatment did not induce any measurable effects in both HRP^- and HRP^+ cells at the concentrations analyzed (data not shown). This anoxic selectivity was lost after prolonged exposure (24 h; Figs. 6*D* and 7*F*), although the 5-Br-IAA-mediated cell kill in HRP^+ cells was higher than that observed after anoxic treatment with any of the other prodrugs tested.

Bystander Effect of the HRP/IAA System. The bystander effect can be defined as the ability of HRP-expressing cells to kill neighboring HRP^- cells in the presence of the prodrug IAA. Bystander killing is crucial for a successful GDEPT strategy because with the protocols currently adopted in clinical trials, the *in vivo* transfection efficiency is unlikely to be equal to 100%.

The bystander effect induced *in vitro* by the HRP/IAA system was examined in T24 cells by exposing populations of HRP^- and HRP^+ cells mixed in varying proportions to IAA, 1-Me-IAA, or 5-Br-IAA for 24 h. The concentration of prodrugs used in these experiments was chosen to be less than one-half the IC_{50} in HRP^- T24 cells, *i.e.,* 0.5 mM IAA, 0.5 mM 1-Me-IAA, and 0.1 mM 5-Br-IAA, which had little or no effect on cell survival in the mock-transfected population. The percentage of HRP^+ cells was assessed by immunostaining. Fig. 8 shows that the three prodrugs were able to induce significant bystander killing in normoxic as well as in anoxic conditions. A total of 60–70% of the cells in the normoxic culture mixture could be killed when only 5% expressed HRP. A cell kill of 80–95% was achieved when 20–25% of cells were transfected with the HRP gene, which was the maximum transfection efficiency achievable in these experiments (Fig. 8).

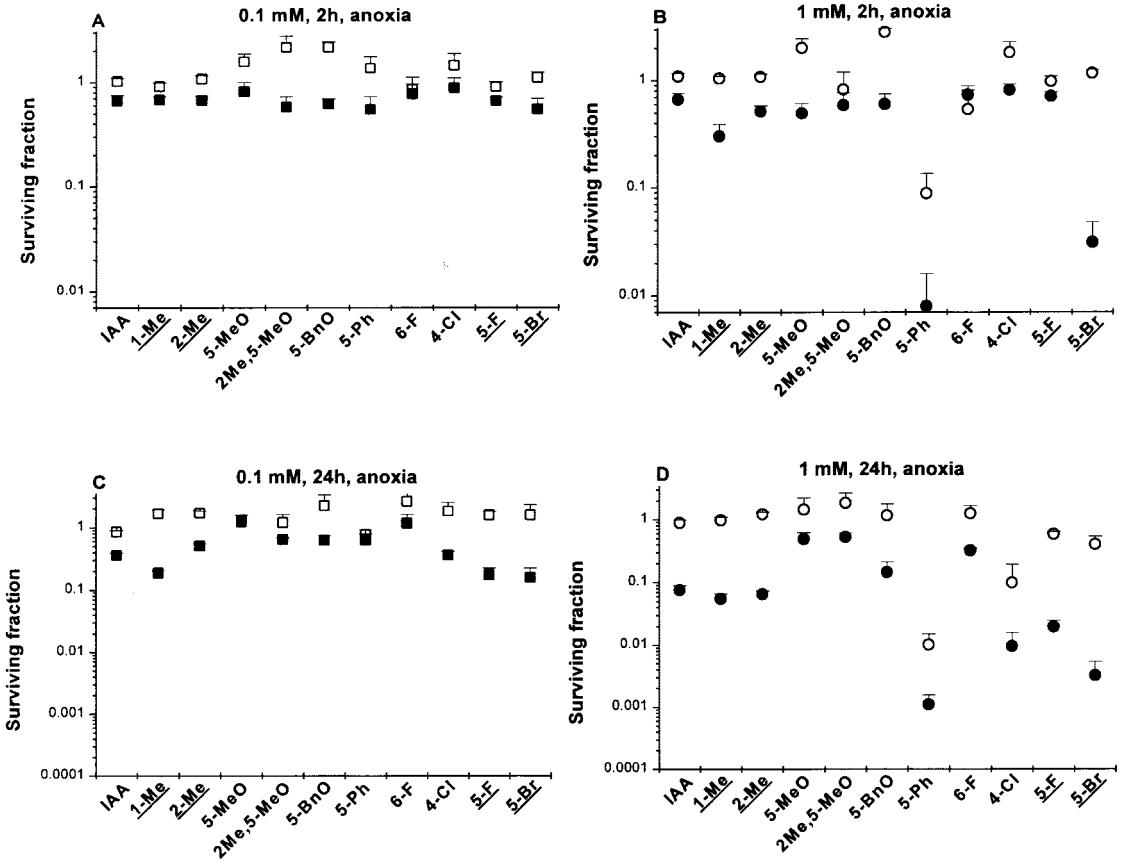


Fig. 5. Use of IAA derivatives for GDEPT in anoxia. Clonogenic survival of T24 cells exposed to IAA derivatives in anoxia at concentrations of 0.1 mM (*A* and *C*) or 1 mM (*B* and *D*) for 2 h (*A* and *B*) or 24 h (*C* and *D*). □, HRP^- cells, 0.1 mM prodrug; ■, HRP^+ cells, 0.1 mM prodrug; ○, HRP^- cells, 1 mM prodrug; ●, HRP^+ cells, 1 mM prodrug. The data are the means of three independent experiments ± SE. Prodrugs adopted in subsequent survival studies are *underlined*.

This effect was not dependent on contact between HRP^+ and HRP^- populations because HRP^- cells were killed when exposed to IAA preactivated by HRP^+ cells (medium-switch experiments), as we have shown previously (4).

An even greater bystander effect was observed in anoxia: 80% (for IAA) to 96% (for 5-Br-IAA) cell kill occurred when only 5% of the exposed population were HRP^+ (Fig. 8), and 2-log killing was induced at the highest concentration of HRP^+ cells (20%). In contrast to what was observed in normoxia, the toxic product generated in anoxia was not transferable in medium-switch experiments (results not shown). However, transfer of cytotoxicity could be detected when the oxygen concentration was raised to 0.1% (data not shown).

Discussion

We have previously shown the potential of the HRP/IAA combination for GDEPT of cancer (4). In the present study, the action of HRP GDEPT was validated in four cell lines of human origin. The response of the three tumor cell lines analyzed, MCF-7 breast adenocarcinoma, FaDu nasopharyngeal carcinoma, and T24 bladder carcinoma cells, did not differ considerably (Fig. 2). After a 24-h incubation with IAA at prodrug levels below 1 mM, HRP expression conferred a slightly higher sensitivity to MCF-7 cells, whereas at prodrug levels above 1 mM, HRP^+ T24 cells were markedly more affected by the treatment. The *p53* status does not appear to play a major role in the response of these tumor cell lines to HRP/IAA because MCF-7 cells have a wild-type *p53* gene (20), whereas FaDu and T24 cells are characterized by *p53* nonsense and missense mutations at codons 248 and 126, respectively (21, 22). HRP/IAA may therefore function efficiently in the many tumors exhibiting mutant p53 phenotypes.

HRP/IAA GDEPT also induced significant inhibition of proliferation in HMEC-1 endothelial cells (Fig. 3*A*). This could represent an advantage if the tumor vasculature was to be targeted. Selective killing of the endothelial cells forming the lining of tumor blood vessels may cause malignant cells to starve for lack of nutrients, producing an amplification of the cytotoxic effects. Also, endothelial cells lack the drug resistance characteristic of neoplastic cells, requiring lower doses of cytotoxic agents, and the vicinity to the blood stream allows direct and simplified agent delivery. For HRP/IAA, low prodrug doses would be used because IAA alone showed some toxicity in HRP^- cells (Fig. 3*A*). Thus far, the effects of

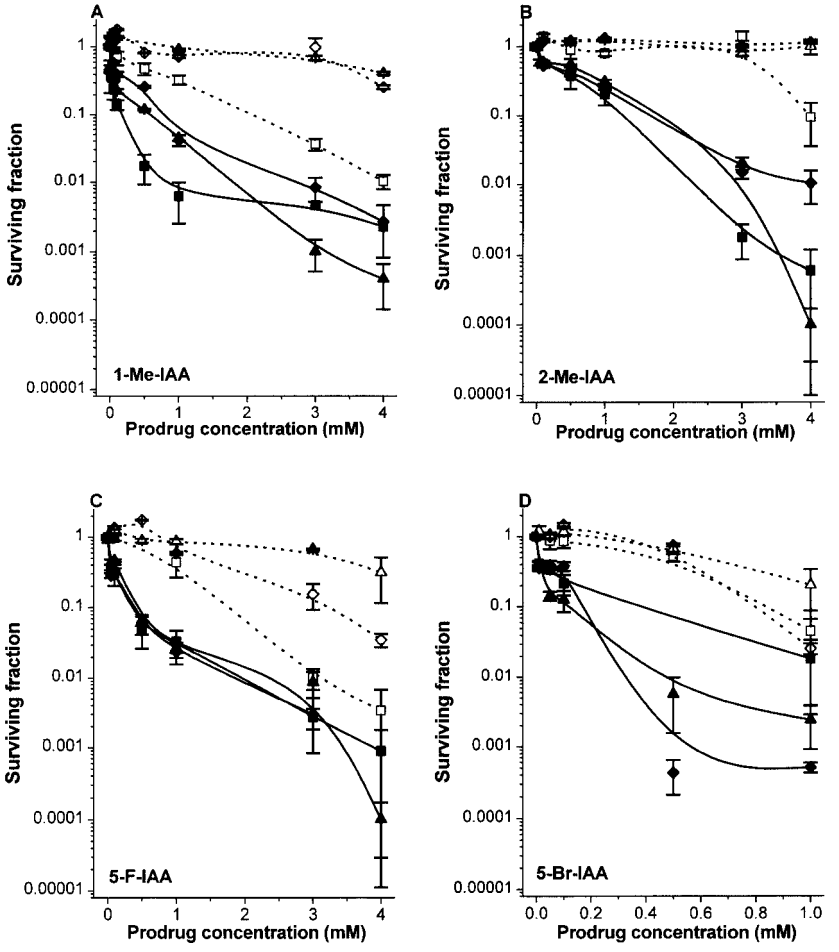


Fig. 6. Cytotoxicity of IAA analogues in normoxia. Clonogenic survival of HRP^- and HRP^+ tumor cells exposed for 24 h to 1-Me-IAA (*A*), 2-Me-IAA (*B*), 5-F-IAA (*C*), and 5-Br-IAA (*D*). △, HRP^- T24 cells; ▲, HRP^+ T24 cells; □, HRP^- MCF-7 cells; ■, HRP^+ MCF-7 cells; ◇, HRP^- FaDu cells; ♦, HRP^+ FaDu cells. Means and SEs of at least three independent experiments are indicated. The lines are interpolated.

HRP/IAA have not been tested in normal cells other than HMEC-1 cells, but preliminary *in vivo* studies indicate very low toxicity of the prodrug and the activated drug in non-cancer tissue.[4]

The possibility of enhancing the antitumor effect of HRP GDEPT was investigated in a panel of different IAA analogues. The screening of 10 IAA derivatives in the four cell lines adopted in this study allowed the identification of compounds characterized by fast normoxic (1-Me-IAA, 2-Me-IAA, and 5-MeO-IAA) or anoxic (5-Br-IAA) cytotoxic activation, high HRP^+ cell kill (5-Br-IAA, 4-Cl-IAA, 5-BnO-IAA, 5-Ph-IAA, 5-F-IAA, and 1-Me-IAA), or selectivity [1-Me-IAA, 5-Br-IAA, and 5-F-IAA (Figs. 4–7)]. Some variations were observed in the response of cells of different origin, with IAA, 1-Me-IAA, and 5-Br-IAA representing the most promising candidates for HRP GDEPT. In particular, 5-Br-IAA showed very prompt and selective anoxic activation (Fig. 7, *E* and *F*). The choice of the appropriate prodrug and dose may therefore depend on the tumor type, the hypoxic fraction, and the pharmacokinetics *in vivo*.

HRP activation of the IAA prodrug in the presence of oxygen leads to formation of a stable toxic product, and the toxicity is transferable to HRP^- cells under normoxia (4, 10) or hypoxia (0.1% O_2; results not shown). However, no such transferability was seen under anoxia (data not shown), indicating that the anoxic toxicity is caused by relatively short-lived reactive species. The initial activation of IAA by HRP generates a radical species (skatole radical), which is followed by a complex series of reactions with several possible pathways involving short-lived radical intermediates and longer-lived species (6). In normoxia, 3-methylene-2-oxindole is likely to be involved in HRP-mediated cell death.[5] Under anoxic conditions, the pathway leading to 3-methylene-2-oxindole would not be available, and other toxic species are likely to be involved, as discussed in Ref. 4. In the case of 5-Br-IAA, the early toxicity after 2 h of incubation in anoxia may be due to an enhanced rate of one of these as yet undetermined reaction pathways, for this particular analogue only. Further work will concentrate on the analysis of the cytotoxic pathways at different oxygen concentrations.

The variation in toxicity of the compounds analyzed does not appear to depend on the rate of oxidation in the presence of HRP because no correlation could be measured between

[4] L. K. Folkes, unpublished data.

[5] S. Rossiter, unpublished data.

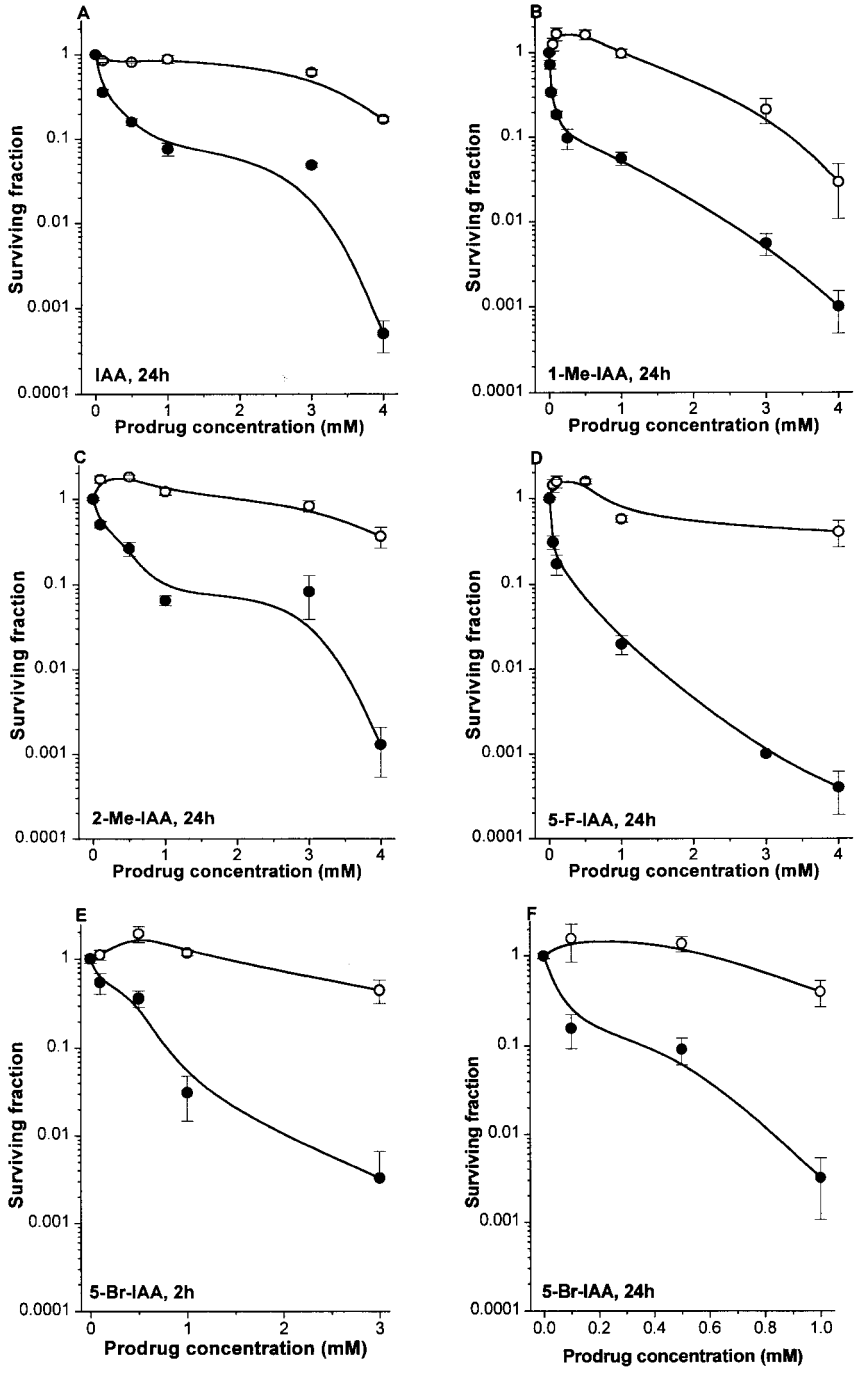


Fig. 7. Cytotoxicity of IAA and analogues in anoxia. T24 cells transiently transfected with the HRP cDNA and mock-transfected cells were exposed for 24 h to prodrugs IAA (*A*), 1-Me-IAA (*B*), 2-Me-IAA (*C*), and 5-F-IAA (*D*) or exposed to 5-Br-IAA for 2 h (*E*) or 24 h (*F*). *A*–*F*: ○, HRP^- cells; ●, HRP^+ cells. The means of three independent experiments ± SE are indicated. The lines are interpolated.

oxidation rate[4] (6) and surviving fraction after a 2-h drug exposure. Unexpectedly, after a 24 h-incubation with HRP^+ cells, the IAA analogues that are not promptly oxidized by HRP (such as 5-F-IAA and 5-Br-IAA) resulted in the highest toxicity. Components and substituent position may both be relevant to the cytotoxic potential, as seen from the different efficacies of 1-Me-IAA and 2-Me-IAA or 5-F-IAA and 6-F-IAA (Figs. 4–7), for example.

Gene delivery to tumors *in vivo* is unlikely to lead to the modification of 100% of the target cells. Therefore, an essential requirement for GDEPT is that the activated drug should induce a bystander effect, whereby conversion of the prodrug to the active form in the enzyme-modified cells leads to the killing of adjacent untransfected ones. The killing of neighboring cells can be due to the transfer of toxic metabolic products through gap junctions (23), via apoptotic vesicles (24), or through the diffusion of soluble toxic metabolites (25, 26). Our studies suggest that the HRP/IAA system can produce a strong bystander effect. In all experiments, HRP transfectants were estimated to represent at best a quarter of the cells exposed to IAA, but this

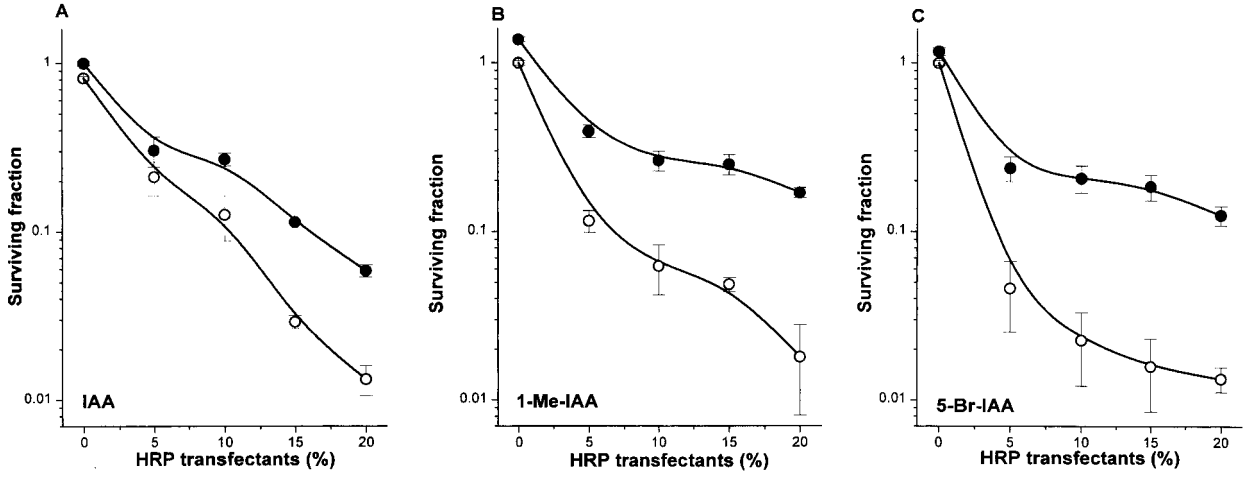


Fig. 8. Bystander effect of the HRP/IAA combination. HRP^- and HRP^+ T24 cells were mixed in the proportions indicated and treated with 0.5 mM IAA (*A*), 0.5 mM 1-Me-IAA (*B*), or 0.1 mM 5-Br-IAA (*C*) for 24 h in normoxia (●) or anoxia (○). Means ± SE are indicated. The lines are interpolated.

mixed population could be almost completely eradicated (Fig. 2). Transient rather than stable lines were used because transient expression and lack of integration of the therapeutic gene are more likely to take place *in vivo* with current viral or nonviral delivery systems (*e.g.,* adenoviruses and cationic liposomes). In mixing experiments, approximately 70% and 90% cell kill was observed, with only 5% and 20% of the cells expressing HRP, respectively (Fig. 8). The effect does not appear to require contact between HRP^- and HRP^+ populations because incubation of HRP^- cells with preactivated IAA resulted in cell death under both normoxia (4) and hypoxia (0.1% O_2). This compares very favorably with *in vitro* data on the bystander cytotoxicity of other enzyme/prodrug systems. For example, 95% cell kill after ganciclovir treatment required expression of herpes simplex virus-1 thymidine kinase in 50% of the exposed population (24). Similarly, expression of cytosine deaminase in 5% of the cells resulted in 50% cell eradication after 5-fluorocytosine (27), and 90% growth inhibition could be achieved when 34–50% of the cells exposed to CB1954 produced the enzyme nitroreductase (26, 28). Importantly, an even more pronounced bystander effect was observed in anoxic conditions (Fig. 8), which, as discussed previously, is likely to be due to short-lived reactive species.

The prodrug concentrations used in the *in vitro* bystander cytotoxicity experiments fall within the range of concentrations that could be achieved *in vivo*. In preliminary *in vivo* studies, 250 mg/kg IAA and 50 mg/kg 5-F-IAA i.p. in mice resulted in tumor prodrug levels of ~1 and ~0.2 mM, respectively, and plasma levels in excess of ~1–3 mM.[6] No adverse effects were recorded in normal tissues. Nevertheless, to achieve prodrug activation at the target only, current work is focused on placing the HRP gene under the control of hypoxia-responsive promoters,[7] which may ensure selective therapeutic gene expression at the tumor site (29).

In summary, HRP represents an effective enzyme for use in combination with the IAA prodrug and its analogues. We are currently investigating the use of this combination for GDEPT *in vivo* in HRP-modified tumor xenografts of the bladder and nasopharyngeal carcinoma cell lines described. The *in vitro* analysis presented here allowed the identification of compounds with potential not only in gene therapy strategies, but also in antibody-directed enzyme/prodrug therapy and polymer-directed enzyme/prodrug therapy approaches. Particular attention will be drawn to the action of HRP in combination with IAA, 1-Me-IAA, and 5-Br-IAA.

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[6] Unpublished observations.

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