Use of horseradish peroxidase for gene-directed enzyme prodrug therapy with paracetamol

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Gene therapy is a potential method of treating cancer with a greater degree of targeting than conventional therapies. In addition, therapy can be directed towards cells within the tumour population that are traditionally resistant to current treatment schedules. Horseradish peroxidase (HRP) can oxidise paracetamol to N-acetyl-p-benzoquinoneimine via a one-electron pathway. Incubation of human cells expressing HRP with 0.5–10 mM paracetamol reduced clonogenic survival, but had little effect on control cells. A small increase in apoptosis was seen and a decrease in the number of cells undergoing mitosis, consistent with reports in hepatocytes using higher paracetamol concentrations. The cytotoxicity was also seen under conditions of severe hypoxia (catalyst induced anoxia), indicating that the HRP/paracetamol combination may be suitable for hypoxia-targeted gene therapy.

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A number of gene therapy strategies have been proposed for the treatment of cancer. One system is gene-directed enzyme prodrug therapy, or GDEPT. Gene-directed enzyme prodrug therapy is a two-step process which involves the delivery of a gene encoding an enzyme, followed by administration of a nontoxic prodrug, which is converted to a cytotoxin by the enzyme. Several GDEPT combinations have been proposed (Springer and Niculescu-Duvaz, 2002). This combination has been shown to improve survival in hypoxic tumours. Hypoxia-selective cytotoxicity of paracetamol combination was capable of producing a cytotoxic effect in hypoxic regions of tumours.

The aim of the current study was to determine whether an HRP/paracetamol combination was capable of producing a cytotoxic species in a GDEPT setting and, in particular, whether it would be suitable for targeting radioresistant hypoxic cells.

MATERIALS AND METHODS

Cell culture

Human nasopharyngeal squamous cell carcinoma cells, FaDu, were obtained from the American Type Culture Collection (Manassa, VA, USA). These cells carry a nonsense mutation within the p53 gene (Reiss et al, 1992). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (Sigma, Poole, UK) and 2 mM L-glutamine (Life Technologies). Cells were kept in a humidified incubator at 37°C and 5% CO₂/air. Cells were routinely sub-cultured in 75 cm² cantilevered flasks.

Cells were transfected with either the HRP gene (pSSHRP-puro, kindly provided by Dr O Greco, Gray Cancer Institute), or the marker green fluorescent protein (GFP, pEGFP-puro) using a...
nonviral method, as previously described (Greco et al., 2000). Stably transfected clones were selected in media containing 1 μg ml⁻¹ puromycin (Sigma), which resulted in the death of parental cells within 72 h, and colonies isolated. Gene expression was confirmed by fluorescent-activated cell sorting (FACS) for GFP or HRP enzyme activity, using a modified TMB assay (Greco et al., 2000). Horseradish peroxidase activity was seen in HRP and not GFP transfectants. Single clones were isolated, named HRP8 and GFP1, and cell lines derived from these initial clones were used throughout the experiments. Horseradish peroxidase activity expressed as units per mg protein were 0.08 ± 0.003 and 0.002 ± 0.01 for HRP8 and GFP1 cells, respectively. Cells were confirmed as mycoplasma negative using a PCR method (ATCC Mycoplasma Detection Kit Version 2.0).

Clonogenic assay
Exponentially growing cells were collected from monolayer culture by trypsinisation and plated at low density. Cells were plated as either GFP1 or HRP8, or a mixture of 50% GFP1 and 50% HRP8 cells. Cells were allowed to adhere for 4–6 h. Prodrugs were dissolved in Hank’s balanced salt solution (HBSS, Life Technologies) and cells were exposed in the 37°C incubator for 4 or 24 h.

Following drug exposure, cells were washed in phosphate-buffered saline (PBS) and grown for approximately 10 days in complete media supplemented with feeder cells (V79 cells exposed to 250 Gγ⁻¹Co). Colonies were fixed in 0.5% methylene blue w/v⁻¹ in isomethylated spirit (IMS). Colonies estimated to be greater than 50 cells were counted, and the survival was expressed relative to vehicle-treated controls.

For experiments conducted under anoxic conditions, cells were plated on oxygen impermeable permox dishes (Nunc), allowed to attach and then moved to an anoxic chamber (Don Whitley Scientific), and media were replaced. After an hour, cells were exposed to prodrug in the chamber. After incubation, medium was removed, cells were washed, and incubated with feeder cells as before. Media and prodrug solutions were kept under anoxia for at least 14 h before addition to cells.

Glutathione measurements
The GSH level in cells was measured using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer’s instructions. Glutathione reacts with Ethman’s reagent to form a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) and a mixed disulphide. The disulphide is reduced by glutathione reductase to recycle the TNB and form further GSH. The rate of TNB production is proportional to the recycling reaction, which is proportional to the concentration of GSH in the sample.

Cells (5 × 10⁴) were collected by scraping and centrifugation. The cell pellet was resuspended and sonicated in phosphate buffer containing 1 mM EDTA. This was centrifuged and the supernatant deposited with an equal volume of metaphosphoric acid (MPA, Aldrich, Poole, UK), centrifugation, and treatment with 50 μl of 4 m triethanolamine per millilitre of sample. Samples were then added to a 96-well plate (TPS), and assay cocktail was added and absorbance at 415 nm was measured every 5 min for 20 min on a Labtech plate reader. GSH levels were calculated by producing a standard curve of known GSH concentrations.

Cell cycle analysis
Cells were exposed to paracetamol as for clonogenic assays. Following 24 h exposure, cells were harvested, and full medium was added for a further 24 h. Cells were then harvested, washed in PBS, fixed in ice-cold 70% ethanol for an hour on ice, rinsed, centrifuged, and the pellet resuspended in a solution containing 20 μg ml⁻¹ propidium iodide, 20 μg ml⁻¹ RNase A (Sigma) in PBS. Cells were incubated at 37°C for 30 min, and then analysed by FACS (Becton Dickson, Cowley, Oxford, UK), gated to exclude cellular debris.

Effect of furafylline on HRP activity
Purified HRP enzyme (0.4 μg, Sigma) was incubated in the presence of varying concentrations of the CYP1A2 inhibitor, furafylline (Sigma), in 80 mM phosphate buffer (pH 5.4). Then, 320 mM 3,3',5,5'-tetramethylbenzidine (TMB) and 3 mM hydrogen peroxide were added to give a total volume of 2 ml. Absorbance was read every minute at 652 nm for 10 min on a Hewlett Packard model 8452A diode array spectrophotometer. Over this period, absorbance increased linearly.

Statistical analysis
JMP statistical analysis programme was used to carry out ANOVA and t-test analyses.

RESULTS
Incubation with paracetamol for 4 h resulted in a decreased clonogenic ability of FaDu cells stably expressing the HRP enzyme (HRP8). Concentrations of 1 mM prodrug resulted in greater than 50% cell kill (Figure 1A). Concentrations greater than 1 mM did not appear to further increase the cell kill. There was no loss of viability in GFP controls up to 8 mM paracetamol.

After 24 h exposure to paracetamol, clonogenic survival of HRP8 cells decreased sharply with increasing concentrations of prodrug, with over a log of cell kill at 10 mM paracetamol (Figure 1C). However, the extended incubation resulted in a loss of GFP1 clonogenicity, although to a lesser extent than HRP8.

To determine whether the HRP/paracetamol combination would be suitable for hypoxia targeting, the clonogenic studies were repeated under anoxic conditions. After both 4 and 24 h exposure to paracetamol (Figure 1B and D), HRP8 cells showed a similar decrease in clonogenic survival as under oxic conditions. The IC₅₀ values (concentration required to decrease the surviving fraction by 50%) did not differ between the two oxygen status experiments at either exposure time.

Exposure of a mixture of HRP8 and GFP1 cells to paracetamol for 24 h under oxic or anoxic conditions (Figure 1) resulted in cell kill that almost overlapped with that seen in experiments with HRP8 only cells, indicating a large bystander effect.

There was no significant difference in either the oxidised or reduced form of GSH between HRP8 or GFP1 cells as determined by the recycling assay (Figure 2). There was a slight but not significant difference between GSH levels in parental FaDu cells and HRP8 and GFP1 clones.

Propidium iodide staining of cells showed a significant increase in the sub-G₁ cell population of HRP8 cells following incubation with paracetamol compared with untreated controls (Figure 3). There was also a small decrease in the number of G₂/S cells indicating a decrease in proliferation. In contrast, there was no statistically significant change in the cycle distribution of GFP1 cells.

In experiments using purified HRP enzyme, incubation with up to 25 μM furafylline (a potent cytochrome P450 1A2 inhibitor) led to a maximum decrease of 20% in the enzyme activity (Figure 4). Concentrations of furafylline required to decrease the enzymatic activity of purified HRP enzyme by half were not attainable as the solubility limit of the drug was reached at 250 μM.
DISCUSSION

The results presented here show the ability of the HRP enzyme to catalyse the conversion of paracetamol to a cytotoxin intracellularly. This enzyme/prodrug combination was effective under both oxic and anoxic conditions, with no loss of activity in the absence of oxygen. In addition, mixtures of HRP-expressing and nonexpressing cells showed almost equivalent levels of cell kill to HRP only experiments, indicating a large bystander effect.

The potential of delivering the HRP gene to activate IAA has been shown previously (Greco et al., 2000). In the present study, paracetamol was used as an alternative prodrug to indoles. Sufficient cytotoxin was produced to decrease the clonogenic ability of HRP transfectants both under oxic and anoxic conditions. This effect appears to be independent of p53, since FaDu cells carry a nonsense mutation at codon 248 (Reiss et al., 1992).

The toxicity of the HRP/paracetamol combination is likely to be a result of NAPQI formation. However, the exact mechanism is currently unclear. The data showed a slight increase in apoptosis, as indicated by an increased sub-G1 peak of treated cells after propidium iodide staining, and also a decrease in the number of cells undergoing mitosis (increased G2/M population). However, this may be supplemented by necrosis, since this is the primary finding in vivo following overdose (Timbrell, 1996), and the decrease in clonogenicity seen following paracetamol exposure could not be fully explained by apoptosis. Further experiments are required to determine the exact route of cell death and mechanism of action.

Paracetamol/NAPQI is able to deplete cellular GSH levels (Adamson and Harman, 1993; Pumford et al., 1997), bind to proteins and DNA (Pumford et al., 1997), increase intracellular calcium concentrations (Tsokos-Kuhn, 1989), cause lipid peroxidation, increase reactive oxygen species (Manov et al., 2002), and has been shown to affect cell cycle progression and

**Figure 1** Clonogenic survival of cells following exposure to paracetamol. (A) 4 h air, (B) 4 h in anoxia (catalyst induced), (C) 24 h air, (D) 24 h anoxia. Data are mean ± s.e.m. three experiments, triplicate samples. ● GFP1 controls, □ HRP8 cells, × mixture of 50% HRP8 and 50% GFP1 cells (mosaic).

**Figure 2** Glutathione concentrations in FaDu cells measured using the recycling assay. ■ FaDu parental cells, □ GFP1 clones, ■ HRP8 clones. Data are mean ± s.d. two experiments, duplicate samples.
be due to a direct two-electron oxidation by CYP enzymes. In the case of activation by HRP via one-electron oxygenation, it is possible that there could be constant recycling of the semiquinone back to paracetamol with little formation of NAPQI, provided there is sufficient GSH or NADPH (preferentially GSH (Potter and Hinson, 1987)). This in itself would lead to oxidative stress, and may increase the susceptibility of cells to damage by ionising radiation. Radiotherapy is a standard treatment for many solid tumours, and its combination with GDEPT strategies is promising (Buchsbaum et al., 1996). In fact, the HRP/IAA combination sensitised cells to radiation in air and hypoxia (Greco et al., 2002b). Hence, future work is aimed at determining whether the HRP/paracetamol combination would act as radiosensitizer.

There was no detectable difference in GSH levels between GFP1 and HRP8 cells, indicating that the increased susceptibility of HRP8 transfectants to paracetamol is unlikely to be due to an exacerbation of an imbalanced oxidative state. It has previously been reported that the addition of HRP to cells can decrease GSH levels (Harman et al., 1986). From our results it appears that the constant production of the enzyme intracellularly has no greater effect on GSH concentrations than the production and persistence of GFP. Overall, however, transfected cells tended to have lower GSH levels than untransfected FaDu cells. This could be due to either the presence of the transgene, or may be a consequence of the presence of puromycin antibiotic in the growth media.

The concentrations of paracetamol used to achieve cell kill are similar to those used by Thatcher et al. (2000) for prodrug activation using CYP1A2. This group showed decreased cell viability following exposure of fibroblasts overexpressing CYP1A2 to paracetamol. The levels used in both studies are greater than those achievable after current therapeutic doses of paracetamol. However, it may be possible to increase paracetamol doses in patients by combining treatment with furafylline, a potent CYP1A2 inhibitor (Sesardic et al., 1990). Plasma steady-state levels of furafylline can reach 5.8 µM in humans (Tarrus et al., 1987), and the IC50 for purified CYP1A2 is 0.07 µM (Sesardic et al., 1990), indicating that the use of furafylline to inhibit paracetamol activation in the liver is feasible. Importantly for our study, furafylline had little effect on HRP enzyme activity up to 25 µM. Orally administered methionine or N-acetyl cysteine could also be administered to increase liver GSH levels (McLean and Day, 1975; Aebi and Lauterburg, 1992). This would decrease the susceptibility of the liver to damage by NAPQI by allowing increased amounts of mercapturic acid to be formed, as well as N-acetylcysteine conjugates. Although somewhat effective at minimising damage following paracetamol overdose, the effect of GSH precursors administered prior to paracetamol in combination with furafylline needs to be assessed.

In conclusion, this data demonstrates for the first time the potential for HRP/paracetamol as a GDEPT strategy, under tumour conditions. Further work needs to be carried out to determine the mechanism of action, the in vivo potential and possible radiosensitising effects before speculating on clinical trial outcome.

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