The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954

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The enzyme nitroreductase, isolated from Escherichia coli B, converts CB1954 ((5-aziridin-1-yl)-2,4-dinitro-benzamide) into a cytotoxic DNA interstrand cross-linking agent. The E. coli B gene (nfnB, NTR) encoding nitroreductase (NTR) was cloned into eukaryotic expression vectors. When driven by a CMV promoter, 5–10% of the stably transfected mouse fibroblasts expressed the NTR enzyme. These cells were killed at a concentration of 20 μM CB1954 in comparison to nonexpressing cells which were killed at a much higher concentration (500 μM). We subsequently generated transgenic mice to test the prodrug system in vivo. Nitroreductase was expressed specifically in T cells driven by the control elements of the human CD2 locus. Upon CB1954 treatment, transgenic mice show extensive cell depletion in thymus and spleen (14–16% of normal cell numbers), whereas all other tissues are unaffected by pro-drug administration. These results raise the possibility of using the NTR gene in anticancer therapy.

Keywords: CB1954; locus control region; nitroreductase; transgene

Introduction

Prodrugs are substances which are not toxic, but can be activated to produce cytotoxic derivatives. For the purposes of anticancer therapy, this activation should be specifically associated with tumour cells. The monofunctional alkylating agent CB1954 (5-aziridin-1-yl)-2,4 dinitrobenzamide) was synthesised in the late 1960s[1] and was found to be highly potent and selective against the Walker 256 rat tumour line.[2] DNA cross-link formation in affected cells was a result of the bioactivation of the drug by the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone)) in the Walker cells which reduces the 4-nitro group of CB1954. The product of this reaction is a difunctional alkylating agent, 5-aziridin-1-yl-4-hydroxylamino-2-nitrobenzamide. This molecule can not cross-link DNA itself, but is further activated by a nonenzymatic reaction with thioesters (such as coenzyme A).[3] However, except for a few rat hepatoma and hepatocyte cell lines, other rat cells are not known to be sensitive to CB1954, in agreement with their low levels of DT diaphorase, and human cells, even those expressing significant levels of the human DT diaphorase, are not sensitive due to differences in the kinetics of CB1954 reduction by these two forms of the enzyme.[4]

Apart from Walker cells, sensitivity to CB1954 has also been reported in bacteria.[5] The toxicity and mutagenicity of CB1954 was greatly reduced in a nitroreductase-

deficient strain of E. coli,[6] suggesting that CB1954 can also be activated by nitroreduction in E. coli in an analagous manner to its bioactivation in Walker cells. A nitroreductase enzyme (NTR) has been isolated from E. coli B.[7] The enzyme is an FMN-containing flavoprotein with molecular mass of 24 kDa and requires either NADH or NADPH as a cofactor. Indeed, it was found that E. coli nitroreductase is capable of reducing CB1954, that it is a more active enzyme (k_{cat} = 360/min for CB1954 versus k_{cat} = 4/min for Walker cell DT diaphorase) and also has a lower K_m for NADH (6 versus 75 μM).[8] Thus E. coli nitroreductase is potentially a suitable candidate for cancer chemotherapy programmes such as antibody-directed enzyme prodrug therapy (ADEPT), where a tumour-selective monoclonal antibody is conjugated to an enzyme that is capable of bioactivating a prodrug.[9] Isolation of the gene encoding nitroreductase, nfnB (NTR)[10] also raises the possibility of gene-directed enzyme prodrug therapy (GDEPT), where a gene is introduced into cells, and if expressed can activate a prodrug. Expression can be directed to the tissue(s) of interest by using an appropriate locus control region (LCR), such as described for the human β-globin gene locus,[11] human CD2,[12] chicken lysozyme,[13] human S100β[14] and mouse MHC class II.[15]

Here, we describe the expression of NTR in eukaryotic cells. Initially, the enzyme was expressed in a mouse fibroblast cell line. Expressing cells could be killed by the application of the prodrug to the culture. To show that the prodrug system works in vivo and exclusively on targeted tissues, we generated transgenic mice expressing nitroreductase in T cells but not in other tissues. Upon prodrug treatment there is a dramatic depletion of cells in the thymus and spleen of transgenic animals, whereas nonlymphoid tissues are normal and comparable with untreated transgenic or wild-type mice.

Results

Transfection of mouse L cells with pCMV/NTR results in expression of nitroreductase in fewer than 10% of cells in culture
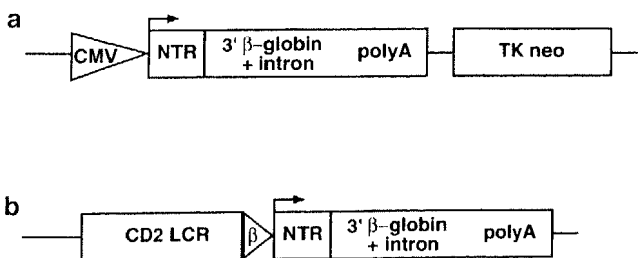
The nitroreductase gene (*nfnB*) was isolated from *E. coli* B as a PCR fragment of approximately 750 bp by N Minton (PHLS, Porton Down, UK). After improving the ribosome binding site and removing an upstream ATG we cloned this fragment into mammalian expression cassettes (see Materials and methods).

Initial tests were done on stably transfected mouse L cells with the construct pCMV/NTR (Figure 1a). After selection for 7 days at 800 μg/ml G418, 10 independent clones were picked and kept further at 400 μg/ml G418.

The clones were analysed for expression at the RNA level. An S1 nuclease protection assay using a 3′ end probe showed a protected fragment of the expected size (213 bp) in all clones (see Figure 2a). Knowing that there is a stable message, we performed indirect immunofluorescence using a rabbit polyclonal antinitroreductase antibody. By counting NTR positive cells in randomly chosen fields, it was estimated that none of the clones expressed nitroreductase in more than 10% of cells. Most of the clones showed fewer than 5% of cells expressing at one time. This phenomenon is often observed when genes are integrated in the DNA of cultured cells and has been discussed but not completely explained. It may be that in the absence of a sufficiently strong enhancer, heterochromatin can spread from flanking areas into the integrated construct leading to extensive silencing of the gene.[16]

CB1954 causes gross changes in morphology and eventual cell death in cells expressing nitroreductase

The fact that only a small percentage of cells were expressing NTR made quantitative analysis of the effect of CB1954 impossible, so we used a qualitative approach. pCMV/NTR transfected L cells were exposed to the prodrug for various times, and prodrug concentrations between 10 μM and 500 μM. Untransfected cells were not affected by CB1954 below a concentration of 500 μM, whereas cells expressing nitroreductase started to show signs of toxicity at 10 μM and were all affected at 20 μM. Effects of the prodrug on cells expressing nitroreductase

a

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--◁CMV▷-- NTR | 3' β-globin + intron | polyA -- | TK neo |--
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b

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--| CD2 LCR |▷β▷| NTR | 3' β-globin + intron | polyA |--
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Figure 1 Nitroreductase expression constructs. (a) pCMV/NTR. The human cytomegalovirus (CMV) immediate–early gene promoter/enhancer was used to drive expression of nitroreductase. To enhance the stability of the mRNA, the 0.75-kb NTR gene was linked to a 2.8-kb fragment from the 3′ end of the human β globin gene, providing the final intron, 3′UTR and polyadenylation signals. (b) pCD2/NTR. To drive expression of nitroreductase specifically in T cells, the NTR gene was coupled to a 2.1-kb HindIII fragment containing the human CD2 locus control region and a β globin promoter fragment of approximately 500 bp.
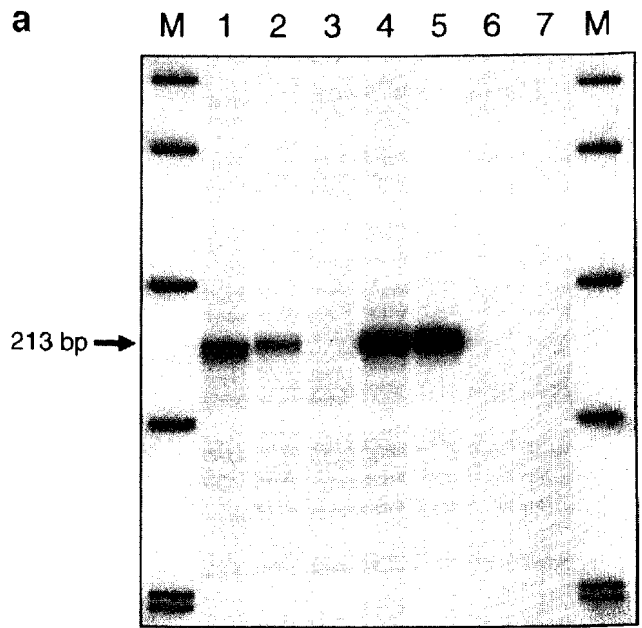
213 bp ➡

Figure 2 Expression of NTR in murine cells. (a) S1 nuclease protection analysis of NTR mRNA in transfected L cells and transgenic mouse thymocytes. The human 3′β globin EcoRI–PstI probe gives a protected fragment of 213 bp after digestion of hybridised RNA with S1 nuclease, due to the presence of 3′ β globin sequences in the constructs. For each sample 10 μg of total cellular RNA was hybridised to approximately 15 ng of ³²P-labelled probe. Lanes 1 and 2 pCMV/NTR transfected L cells, clones 9 and 10; lane 3, untransfected L cells; lanes 4 and 5, thymocytes from CD2/NTR 14 transgenic mice; lanes 6 and 7, thymocytes from non-transgenic mice; M, marker pUC19 cut with MspI and end-labelled with ³²P.

are gross changes in morphology, including 10- to 20-fold enlargement of the cells, enlargement of nuclei, multiple nuclei and ultimately nuclear disintegration (Figure 3). After 7 days of exposure to the prodrug, these cells disappear from the culture. All cells expressing nitroreductase were affected in this way, whereas nonexpressing cells in the same culture appeared to be normal.

The effect of the prodrug on the cells was irreversible after 2 days of treatment, since returning these cells to normal culture conditions did not lead to their recovery.

CD2/NTR transgenic mice express nitroreductase specifically in T cells and thymocytes

Five founder mice carrying the CD2/NTR construct (Figure 1b) were obtained, and lines of each were established. Different tissues were tested for expression of the transgene by S1 nuclease protection analysis (data not shown). The transgene expression, when detected (two out of five lines) was confined to the expected cell types: T cells and thymocytes (see Figure 2a).

Further experiments were done on line 14 which has three copies of the transgene and the highest level of nitroreductase enzyme as determined by Western blotting (data not shown). FACS analysis on single cell suspensions from thymus showed that all CD2-positive cells express NTR, but that there are two populations of cells: high and low expressors (Figure 2b). The level of NTR expression correlates with that of CD2. In the spleen, NTR is expressed only in T cells. Although CD2 positive in the mouse, B cells do not express the transgene due to the use of the human CD2 LCR. Transgene expression was detected only in B220⁻, IgM⁻, CD2⁺ and CD3⁺ cells.
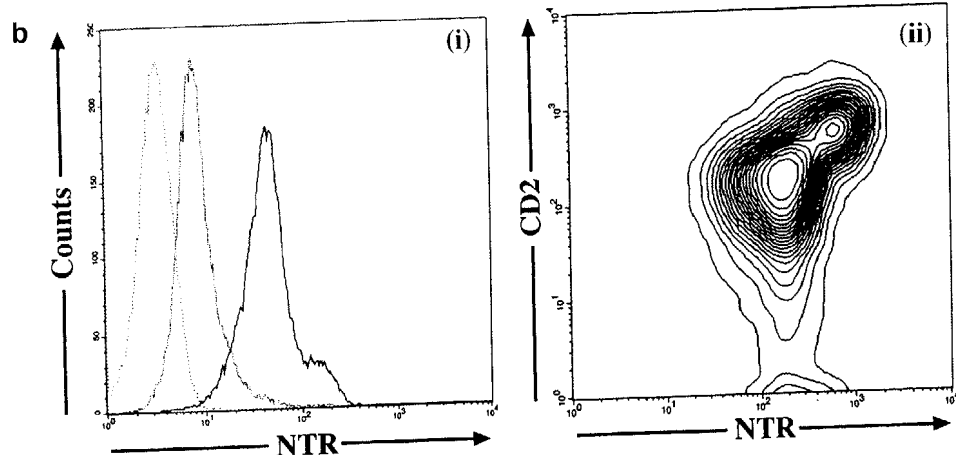
Figure 2 (b) FACS analysis of NTR expression in transgenic mouse thymus. (i) Single-staining with anti-nitroreductase polyclonal antibody on thymocytes from: CD2/NTR transgenic mouse, secondary antibody only (dotted line); non-transgenic mouse, primary and secondary antibodies (dashed line); CD2/NTR transgenic mouse, primary and secondary antibodies (solid line). (ii) Double staining of CD2/NTR transgenic mouse thymocytes with antinitroductase (FITC) and anti-CD2 (phycoerythrin, PE) antibodies.

CD2/NTR transgenic mice show depletion of T cells upon CB1954 treatment

In a pilot experiment, nontransgenic mice (FVB) were injected i.p. with 10, 20 and 50 mg/kg/day CB1954 (dissolved in 20% DMSO in PBS) for 5 consecutive days. These doses were chosen after considering the toxicology and pharmacokinetic data determined for BALB/c and C3H/He mice.[17-19] All animals receiving the highest dose were very sick with multiple organs affected. In particular the intestine (containing NTR-positive bacteria) was severely affected. The lowest dose had absolutely no effect, whereas the middle dose caused a slight reduction in weight compared to nontreated animals but no other visible problems. Hence we chose this dose of 20 mg/kg/day to test the transgenic animals.

Three-week-old transgenic and wild-type mice were injected i.p. with CB1954, solvent only (10% DMSO in PBS), or not injected at all. Two nontreated groups consisted of seven mice each, two solvent-treated groups consisted of nine mice each and two CB1954-treated groups consisted of 13 mice each. Animals received 20 mg/kg of CB1954 per day for 5 consecutive days. Three hours after the last injection mice were killed, different organs macroscopically examined and red and white blood cell counts determined.

Visually, there was an obvious difference in the size of two organs: thymus and spleen. Thymuses and spleens from CB1954-treated transgenics were much smaller than in all other controls. Single cell suspensions were made from these two organs and the total number of cells counted. CB1954-treated transgenic thymuses and spleens had 14-16% of the cell numbers present in controls (Figure 4a).

An assay for apoptosis was performed on single cell suspensions from the thymus using the In Situ Cell Death Detection kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany). A significantly higher percentage of apoptotic cells was seen in CB1954-treated transgenic cells as compared to control groups: 15% versus 0.5-4% (Figure 4b). FACS analysis on thymocytes showed that there is no change in the normal ratio between CD4CD8 double positive, CD4CD8 double negative and single CD4 and CD8 positive cells in treated transgenic mice

(data not shown). Surprisingly, in the spleen the B to T cell ratio is not significantly changed, implying that B cells are also killed.

The red blood cell count was normal and comparable to controls. The white blood cell count was slightly decreased, as expected.

In another experiment, animals were killed 5 days after the last prodrug or solvent treatment instead of 3 h later. It was seen that thymuses and spleens returned to their normal size during this prodrug-free period (data not shown). We therefore conclude that the progenitor cells which do not express CD2 (or the transgene) have not been affected and are able to repopulate the spleen and thymus.
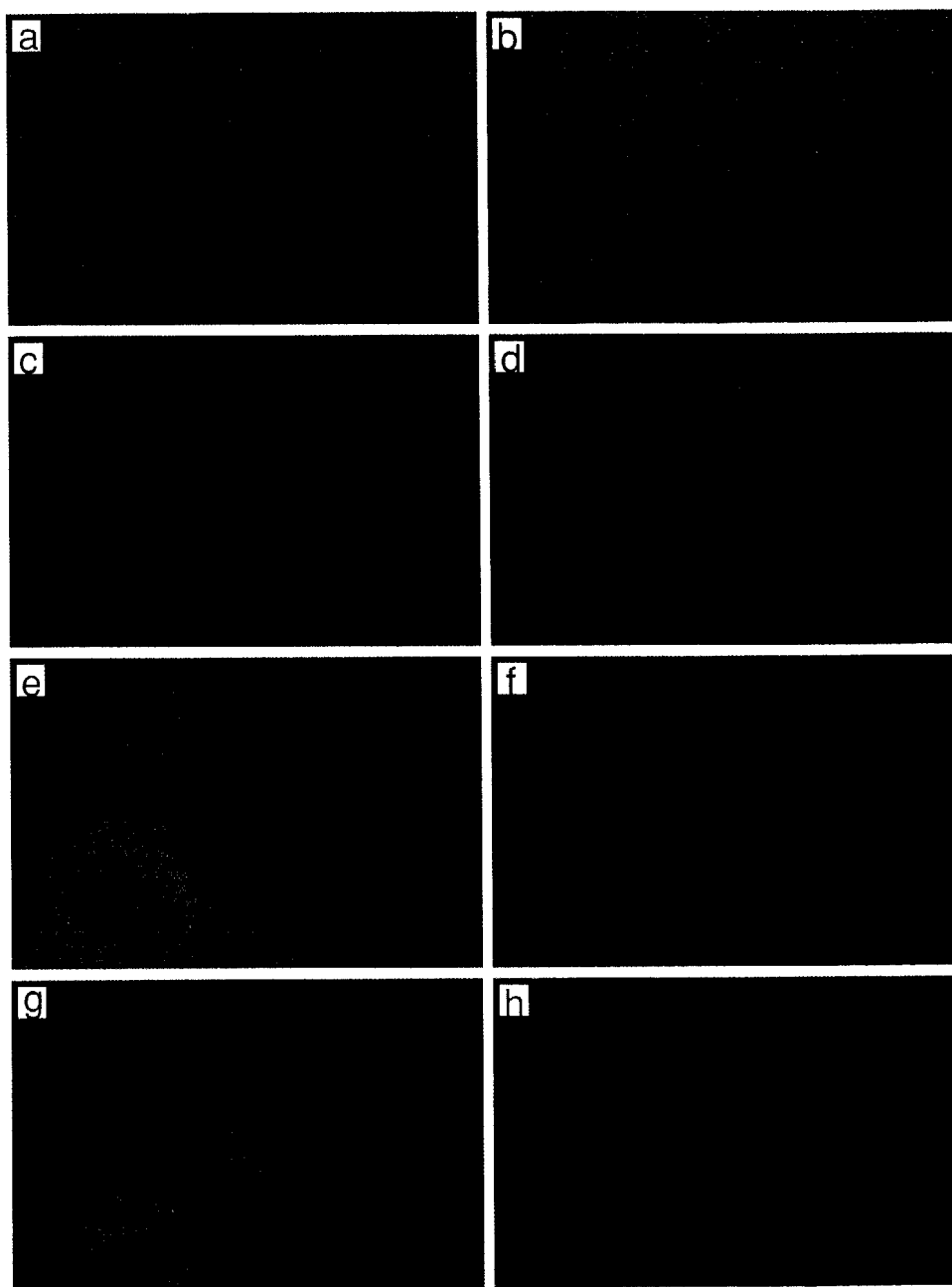
Discussion

CB1954 is converted by nitroreduction from a monofunctional to a difunctional agent. Induced DNA interstrand cross-links are formed with very high frequency, and can contribute up to 70% of total lesions.[20] Interstrand or intrastrand cross-link formations are generally accepted as the most toxic lesions, inhibiting DNA function and eventually resulting in cell death.

Our results showed that eukaryotic cells are able to express the E. coli nitroreductase gene and judging by the end result (cell death), are capable of reducing the substrate (CB1954), inducing DNA interstrand crosslink formation leading to cell death. Changes in morphology of the cells expressing the enzyme upon CB1954 exposure can be explained by a block in DNA synthesis coinciding with a cessation of cell division, although RNA and protein synthesis continue, as evidenced by a continued increase in cell mass. A similar phenomenon was observed in E. coli B_{s-1}.[5]

Our data show that some L cells expressing nitroreductase were able to survive exposure to 10 μM but not 20 μM CB1954, suggesting that at a certain concentration they were no longer capable of repairing the DNA adducts formed as a result of the bioactivation of the prodrug. Cells failed to replicate their DNA and proceed through successive cycles of cell division.

It has been shown that CB1954 forms DNA interstrand

Figure 3 *Treatment of NTR transfected mouse L cells with CB1954. L cells stably transfected with pCMV/NTR and untransfected L cells were treated with CB1954 for various periods of time and at various concentrations. After 5 days, cells were removed from the cultures and analysed for expression of NTR. Cells were stained, mounted and photographed using a ×63 objective lens. (a, c, e, g) Indirect immunofluorescence using a rabbit polyclonal antibody against nitroreductase, with FITC-conjugated goat-anti rabbit Ig as secondary antibody. (b, d, f, h) Nuclear staining of the same fields of cells with 4'-6-diamidino-2-phenylindole (DAPI). (a, b) L cells transfected with pCMV/NTR and grown in the absence of CB1954. (c, d) Untransfected L cells grown in the presence of 50 μM CB1954 for 5 days. (e, f) pCMV/NTR transfected L cells grown in presence of 50 μM CB1954 for 5 days. (g, h) pCMV/NTR transfected L cells grown in the presence of 50 μM CB1954 for 2 days and then in prodrug-free medium for a further 3 days.*

cross-links in Walker cells but not in the insensitive Chinese hamster V79 cells. Co-culturing V79 cells with Walker cells in the presence of CB1954 results in sensitisation of the V79 cells towards CB1954.[21] The toxic metabolite was shown to be soluble and diffusible by its ability to be cytotoxic, and to cross-link DNA in V79 cells. We were expecting to see a similar effect in cell culture experiments. The effect of the prodrug on NTR nonexpressing cells due to the presence of NTR expressing cells in the very same culture was not seen. This could be explained

by the very small percentage of cells expressing the enzyme (5–10%), in agreement with the results of Bridgewater *et al*[22] who found that 30–50% of NTR positive cells are required for 90% overall cell killing, using transduced NIH 3T3 cells. The relatively short half-life of the active metabolite, a lack of intimate cellular contact between expressing and nonexpressing cells or L cells being intrinsically more resistant to CB1954 than V79 cells are other possibilities.

In order to assess the proposed therapy system *in vivo*
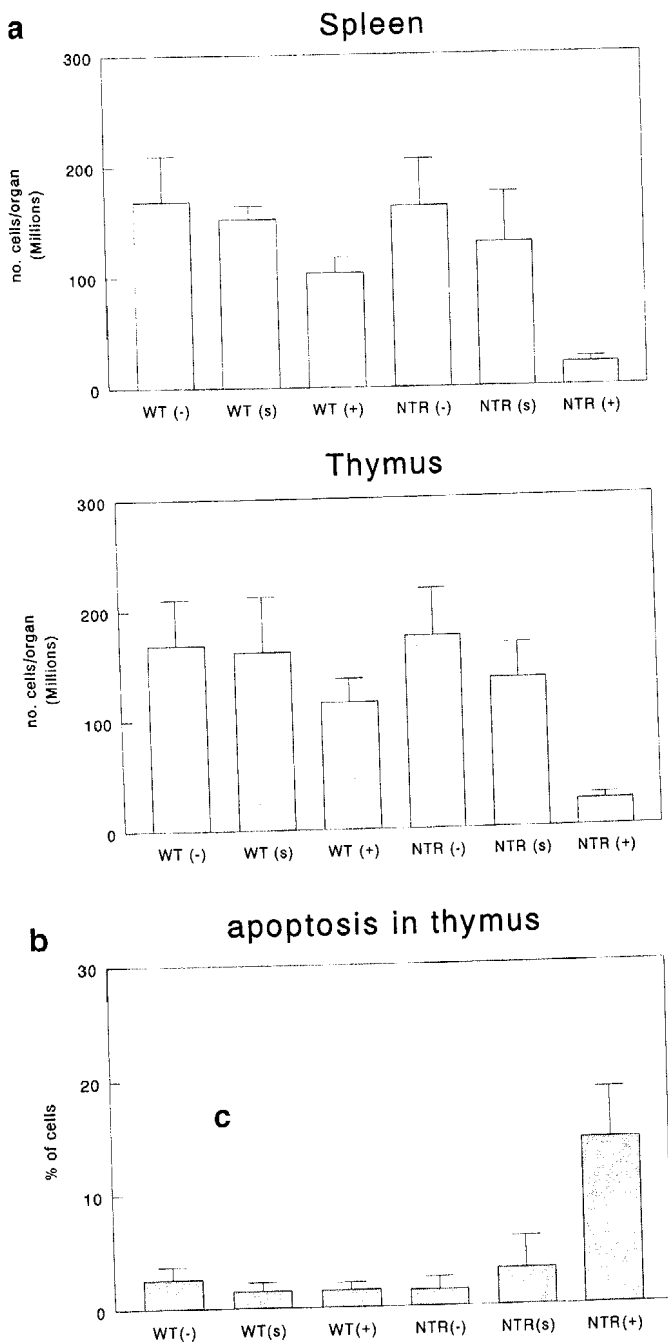
Figure 4 Reduction in cell numbers and an increase in apoptosis is seen after treatment of CD2/NTR transgenic mice with CB1954. (a) Cell numbers. Thymuses and spleens from transgenic and nontransgenic mice were made into single-cell suspensions and counted on a Coulter cell counter. (b) Apoptosis. Cells from the thymuses of treated and untreated mice were assayed for apoptosis using the In Situ Cell Death Detection Kit (Boehringer) which labels breaks in DNA, such as occur during apoptosis, with FITC. The percentage of positively labelled cells was determined using Cell Quest version 2.0 (Graphpad Software, San Diego, CA, USA). NTR, CD2/NTR transgenic mice; WT, nontransgenic mice; (−) no treatment; (s) treatment with solvent only (10% DMSO in PBS); (+) treatment with CB1954 at 20 mg/kg/day. The confidence limits were determined using a two-tailed Student t test, with P = 0.05.

an animal model was tested. The aim was to direct the expression of NTR to a specific tissue and to achieve killing of the chosen target. We directed NTR expression to T cells by the use of human CD2 LCR elements, previously shown to be sufficient for T cell-specific, copy number-dependent, integration site-independent expression in transgenic mice.[23] The transgenic mice which were generated express the nitroreductase in T cells, as predicted. However, it was not expressed in all of the transgenic lines, which may indicate that the 2.1 kb 3′ LCR fragment of the CD2 gene is not sufficient for integration-independent expression of the transgene. Recently, an improved version of a human CD2 mini-gene-based vector has been made for T cell-specific expression in transgenic mice which in addition to the 3′ LCR includes 5′ sequences and the first intron of the CD2 gene.[24] CD2 is one of the earliest markers to appear on the surface of developing thymocytes and in man it is present only on thymocytes and circulating T cells. In the mouse, however, it is present on both T and B cells. As we have used human CD2 locus control elements, expression of the transgene is detected only in T cells.

Toxicity studies on CB1954 were carried out to establish acceptable doses for single and multiple administration in BALB/c and C3H/He mouse strains.[18] Our pilot experiments indicated that the FVB mouse strain used in this study is somewhere between BALB/c and C3H/He in its sensitivity towards CB1954. We used two-thirds of the LD_{10} for a multiple-dose regimen which was established in BALB/c mice (31 mg/kg/day). Weight loss due to enteritis would be the first sign of toxicity, as previously described in rats[2] and in mice.[18] More than half of our experimental animals started to lose weight on day 4 of the experiment, and when killed on day 5 these showed a loss of 5 to 15%, suggesting that the threshold of toxicity was reached. The toxic effect is seen in the spleen of wild-type mice treated with the prodrug where, although the appearance is normal, there is a slight reduction in cell numbers when compared with untreated mice. Since the prodrug was administered intraperitoneally, the spleen was accessible almost immediately after the injection and will have experienced a much higher local concentration of prodrug than the thymus. Nevertheless, our results clearly demonstrate selective killing in lymphoid organs of CD2/NTR transgenic mice. As mentioned before, in fibroblast transfection studies it has been shown that 30–50% expressing cells are enough to kill 90% of all cells.[22] In the spleen, T cells contribute about 40% of the total number of cells, and we speculate that the reduction in B cell numbers is due to a similar (bystander) effect *in vivo*.

It was previously shown that several different chemotherapeutic agents can trigger the apoptotic process.[25] Apoptosis, by morphological criteria, involves chromatin condensation and margination, cell shrinkage, membrane blebbing and formation of nuclear fragments or apoptotic bodies. To see whether CB1954 induces apoptosis in the thymus of transgenic CD2/NTR mice, a TUNEL assay was performed. The results show that only the activated prodrug induces apoptosis in thymocytes.

By using transgenic mice we have shown that only cells expressing the NTR enzyme are able to convert the nontoxic prodrug CB1954 to a cytotoxic drug, resulting in highly specific killing of T cells and thymocytes, while other tissues are unaffected. The apparent killing of NTR

negative B cells in the spleen may result from the high proportion of expressing cells and the proximity of expressing and nonexpressing cells in this organ. The specific killing by nitroreductase/CB1954 is in contrast with cytotoxic agents used in chemotherapy, which in addition to killing tumour cells also affect normal tissues. Nitroreductase is therefore an excellent candidate for gene therapy against tumours. GDEPT has a number of advantages over ADEPT (where the enzyme itself is delivered to cells). The enzyme requires a cofactor (NADH or NADPH) which is present only inside the cell, being rapidly oxidixed and degraded by serum enzymes.[26] Therefore the enzyme needs to be present inside the cell before it can activate the prodrug. Also, specificity can be introduced both by targeted delivery of the gene to the required tissue, and by using tissue-specific elements (LCRs) to drive expression of the enzyme only in the target cells.

Materials and methods

Plasmids (constructs)

The plasmid pJG-1 was constructed by ligation of a 6.1 kb *NotI–ScaI* fragment from PEV-3 (Clare Gooding, Biotechnology Dept, Zeneca, Macclesfield, UK) and a 1.4 kb *NotI–ScaI* fragment from pRc/CMV (Invitrogen BV, Leek, The Netherlands). The resulting plasmid carries a CMV promoter upstream of a multiple cloning site, an intron with RNA splicing sites and a polyadenylation signal from the human β globin gene, β lactamase, and the neomycin resistance gene driven by a HSV-TK promoter. The plasmid pJG-1/NTRΔNotI was constructed by inserting a 0.8 kb *Hind*III fragment carrying the nitroreductase gene from pPM 26 (kindly provided by N Minton) into the *Hind*III site of pJG-1, followed by deletion of a 140 bp *NotI* polylinker fragment between the NTR gene and the splice/polyA site. The final pCMV-NTR construct (Figure 1a) used in tissue culture experiments was created by the removal of a *Hind*III–*Eco*RV fragment from pJG/NTRΔNotI and replacement by an oligo (aagcttgcggccgcgccgccagccatggatatc), thereby (1) removing an extra upstream ATG; (2) inserting a consensus ribosome binding site; and (3) introducing a new *NotI* site. A 0.8-kb *NotI* fragment from pCMV/NTR containing the nitroreductase gene was subcloned into the *NotI* site of PEV-3 and a 7.3 kb *ClaI–ScaI* fragment from the resulting plasmid was ligated to a 3.4-kb *ClaI–ScaI* fragment from GSE 1502b (a 2.1-kb *Hind*III fragment of the human CD2 LCR in pBluescript KS') giving the final pCD2/NTR construct (Figure 1b) used to generate the transgenic mice.

Cell culture, transfection

Mouse L cells were seeded in 10 cm dishes at 1.5×10^6 cells per dish in DMEM/10% FCS. The next day, 3 ml of OPTIMEM-1 (GibcoBRL Life Technologies, Paisley, UK), containing 50 μg of the plasmid pCMV/NTR linearised with *Sca*I was mixed with 3 ml of OPTIMEM-1 containing 100 μl of Lipofectin (GibcoBRL) and added to the cells. Five hours later, 3 ml of DMEM/20% FCS was added to each dish. The next day, the medium was removed and replaced with DMEM/10% FCS containing G418 at a concentration of 800 μg/ml. After 7 days of selection several independent clones were picked and analysed for

expression at the RNA and protein level. For further growth the G418 concentration was redued to 400 μg/ml.

CB1954 assay

Cells were seeded at 1×10^5 cells per dish in 10 cm dishes containing sterile glass coverslips, in triplicate. CB1954 was dissolved in DMSO (100 mM stock) and added to the cells at a final concentration of 0, 10, 20, 50, 100, 250 and 500 μM in DMEM medium. Cells were observed for a period of 7 days. As a control untransfected L cells were used under the same conditions. After 2 days a medium change was performed and half the cultures received the original concentration of the prodrug and the other half were grown in prodrug-free medium. Coverslips with cells attached were removed from the culture at day 5 and immunocytochemistry was performed.

Immunofluorescence

The rabbit polyclonal anti-nitroreductase antibody was kindly provided by R Melton and N Michael, Porton Down, UK. Goat anti-rabbit Ig-FITC was purchased from Sigma (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

In situ staining: Cells were fixed in 3.7% paraformaldehyde (PFA) in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.0, 3 mM MgCl₂, 1 mM EGTA) for 10 min, washed twice and permeabilised for 15 min in 0.5% Triton X-100 in CSK buffer. After two washes in PBS/TWEEN 0.02%, cells were incubated with the anti-nitroreductase antibody (1:200) for 30 min, washed in PBS/TWEEN 0.02% again and incubated with the goat anti-rabbit Ig-FITC antibody (1:100) for 30 min. The cells were washed, the coverslips mounted in DAPI/DABCO/glycerol and analysed under the fluorescence microscope (Leitz).

FACS protocol: A single cell suspension was made in ice cold phosphate-buffered saline pH 7.4 (PBS). Cells were washed in PBS/1%BSA and aliquoted at 10^6 cells per well in a 96-well plate. Cells were first stained with a PE-conjugated anti-CD2 monoclonal antibody (PharMingen, San Diego CA, USA) diluted 1:60 in 50 μl for 10 min at room temperature (RT). They were then washed and fixed in 100 μl 2% PFA for 30 min at RT. Cells were washed in PBS/1%BSA and permeabilised in 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. After a PBS/1%BSA wash, the cells were incubated with the anti-NTR antibody at 1:100 dilution in PBS/1%BSA for 15 min at RT (10^6 cells per 50 μl in 96-well plate). Cells were washed again and incubated with the goat anti-rabbit Ig-FITC antibody at 1:100 dilution for 15 min at RT (10^6 cells per 50 μl). Cells were washed in PBS/1%BSA, resuspended in 200 μl of PBS/1%BSA/0.02% NaAzide and analysed on a FACS analyser (Becton Dickinson, Sunnyvale, CA, USA). For detection of cell surface markers the following monoclonal antibodies were used on unfixed cells: anti CD2-PE (PharMingen), anti-CD3-FITC (PharMingen), anti-CD4-PE (Becton Dickinson, anti-CD8-biotin,[27] anti-B220-cychrome (PharMingen), anti-IgM-biotin (PharMingen). Cells were incubated for 30 min on ice, washed and incubated where appropriate for 30 min with secondary antibodies (tricolor or PE conjugated streptavidin; Caltag Laboratories, Burlingame, CA, USA).

RNA extraction and S1 protection analysis

Total cellular RNA was extracted as described.[28] Specific hybrid message was detected by S1 nuclease protection analysis using a ^{32}P end-labelled DNA probe (700 bp EcoRI–PstI fragment from the human β globin gene) protecting 213 nucleotides. Each hybridisation consisted of 10 μg total RNA and 10–20 ng of probe in a reaction volume of 20 μl. After denaturation at 90°C for 5 min, hybridisation was performed at 53°C for at least 16 h. Subsequent digestion with 100 U S1 nuclease (Boehringer Mannheim) was for 2 h at 25°C in a final volume of 270 μl.

Generation of CD2/NTR transgenic mice

A 6-kb XbaI fragment was purified from plasmid sequences by gel electrophoresis, prepared for injection as previously described[29] and injected into fertilised oocytes (FVB × FVB) at a concentration of 4 ng/μl. Injected eggs were transferred into pseudopregnant mice and transgenic offspring identified by Southern blot analysis of tail DNA.[30]

In vivo CB1954 protocol

Three-week-old mice (heterozygous for the transgene, and nontransgenic FVB) were injected i.p. with CB1954 for 5 consecutive days at 20 mg/kg/day. CB1954 was dissolved in 10% DMSO in PBS. Control animals from both groups were injected with solvent only, or not injected at all. Mice were killed 3–4 h after the last injection. Single cell suspensions were made from thymuses and spleens in PBS as described.[31] The total number of cells per organ was determined using a Coulter cell counter.

Detection of apoptotic cells

1–2 × 10^{6} cells were washed twice in PBS/1% BSA in a V-bottomed 96-well plate. Cells were fixed in 2% paraformaldehyde in PBS, permeabilised with 0.1% Triton X-100/0.1% sodium citrate and labelled by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) reaction using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). Apoptotic cells were detected by flow cytometry (FACS analyser, Becton Dickinson).

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Figure 3 Treatment of NTR transfected mouse L cells with CB1954. L cells stably transfected with pCMV NTR and untransfected L cells were treated with CB1954 for various periods of time and at various concentrations. After 5 days cells were removed from the cultures and analysed for expression of NTR. Cells were stained, mounted and photographed using a ×63 objective lens, (a, c, e, g) Indirect immunofluorescence using a rabbit polyclonal antibody against nitroreductase, with FITC conjugated goat anti-rabbit Ig as secondary antibody (b, d, f, h) Nuclear staining of the same fields of cells with 4′,6-diamidino-2-phenylindole (DAPI). (a, b) L cells transfected with pCMV NTR and grown in the absence of CB1954. (c, d) Untransfected L cells grown in the presence of 50 μM CB1954 for 5 days. (e, f) pCMV NTR transfected L cells grown in presence of 50 μM CB1954 for 5 days. (g, h) pCMV NTR transfected L cells grown in the presence of 50 μM CB1954 for 2 days and then in prodrug free medium for a further 3 days.

cross-links in Walker cells but not in the insensitive Chinese hamster V79 cells. Co-culturing V79 cells with Walker cells in the presence of CB1954 results in sensitisation of the V79 cells towards CB1954.[?] The toxic metabolite was shown to be soluble and diffusible by its ability to be cytotoxic, and to cross-link DNA in V79 cells. We were expecting to see a similar effect in cell culture experiments. The effect of the prodrug on NTR nonexpressing cells due to the presence of NTR expressing cells in the very same culture was not seen. This could be explained by the very small percentage of cells expressing the enzyme (5-10%), in agreement with the results of Bridgewater et al[?] who found that 30-50% of NTR positive cells are required for 90% overall cell killing, using transduced NIH 3T3 cells. The relatively short half-life of the active metabolite, a lack of intimate cellular contact between expressing and nonexpressing cells or L cells being intrinsically more resistant to CB1954 than V79 cells are other possibilities.

In order to assess the proposed therapy system *in vivo*