ORIGINAL ARTICLE

Modified vaccinia virus Ankara as a vector for suicide gene therapy

P Erbs, A Findeli, J Kintz, P Cordier, C Hoffmann, M Geist and J-M Balloul

Transgene S.A., 11 rue de Molsheim, Strasbourg Cedex, France

Modified vaccinia virus Ankara (MVA) has been used successfully to express various antigens for the development of vaccines. Here we show that MVA can also be used as an efficient vector for the transfer of suicide genes to cancer cells. We have generated a new and highly potent suicide gene, *FCU1*, which encodes a fusion protein derived from the yeast cytosine deaminase and uracil phosphoribosyltransferase genes. We now describe the therapeutic benefit of using MVA to deliver and express the *FCU1* gene in cancer cells. MVA-mediated transfer of the *FCU1* gene to various human tumor cells results in the production of a bifunctional intracellular enzyme, such that exposure to the prodrug 5-FC suppresses the growth of the tumor cells both *in vitro* and *in vivo*. Moreover, we report a more potent tumor growth delay at lower doses of 5-FC using MVA-*FCU1* in comparison to adenovirus encoding *FCU1*. Prolonged therapeutic levels of cytotoxic 5-FU were detected in tumors in mice treated with both MVA-*FCU1* and 5-FC while no detectable 5-FU was found in the circulation. This original combination between MVA and *FCU1* represents a potentially safe and attractive therapeutic option to test in man.

Cancer Gene Therapy (2008) 15, 18–28; doi:10.1038/sj.cgt.7701098; published online 9 November 2007

Keywords: MVA; suicide gene; *FCU1*; 5-FC; 5-FU

Introduction

Prodrug-mediated ablation of transduced target cells is now a widely investigated approach for the selective chemotherapy of malignant tumors. Currently, one of the most efficient and extensively studied prodrug activation systems is the bacterial cytosine deaminase (CDase) in combination with 5-fluorocytosine (5-FC).^{1–7} CDase, an enzyme present in fungi and bacteria^{8,9} but absent in mammalian cells,¹⁰ deaminates the nontoxic prodrug 5-FC to its highly toxic derivative 5-fluorouracil (5-FU).

The cytotoxicity of 5-FU is determined by its conversion into 5-fluorouridine triphosphate (5-FUTP) and 5-fluoro-deoxyuridine monophosphate (5-FdUMP). 5-FUTP inhibits RNA synthesis whereas 5-FdUMP inhibits the enzyme, thymidylate synthase, thus depleting replicating cells of thymidine nucleotide precursors during DNA synthesis. The rate-limiting step in the generation of these active species is the formation, via a series of enzymatically catalyzed reactions, of an intermediary metabolite, 5-fluorouridine monophosphate (5-FUMP).

In *Escherichia coli* and *Saccharomyces cerevisiae* 5-FU is converted to 5-FUMP by uracil phosphoribosyltransferase (UPRTase)^{11,12} and it has been reported that adenovirus-mediated transduction of the *E. coli upp* gene

that encodes UPRTase markedly sensitized tumor cells to 5-FU *in vitro* and *in vivo*.¹³

We had previously reported the efficacy of a new suicide gene derived from a fusion of the *S. cerevisiae* CDase (*FCY1*) and UPRTase genes (*FUR1*).¹⁴ This suicide gene, designated *FCU1* (GenBank Accession Number AF312392), encodes a bifunctional chimeric protein that combines the enzymatic activities of *FCY1* and *FUR1* and efficiently catalyzes the direct conversion of 5-FC into the toxic metabolites 5-FU and 5-FUMP.¹⁴ The UPRTase activity of *FCU1* is comparable to that encoded by the parental *FUR1* gene and the CDase activity is increased at least 100-fold over native yeast CDase.¹⁴ Furthermore, this strategy generates a bystander effect, since 5-FU generated within the cells diffuses from the transduced cells into the nontransduced cells.^{14,15} This is distinct from some other prodrug systems which rely on gap junctions for cell-to-cell transfer of the active drug.^{16,17}

Initially, replication-deficient adenovirus was used as the gene transfer vector for *FCU1* and we showed that adenovirus-mediated transfer of *FCU1* to tumor masses derived from human colon carcinoma cells in nude mice suppresses the growth of the tumors when the animals are exposed to systemic administration of 5-FC.¹⁴ Nevertheless, relatively large doses of 5-FC were required for this effect. In an effort to improve the efficacy of the *FCU1*/5-FC strategy for cancer gene therapy, the functionality of the *FCU1* gene was also assessed in a propagation-deficient vaccinia viral context. Modified vaccinia virus Ankara (MVA) is a highly attenuated

Correspondence: Dr P Erbs, Transgene S.A., 11 rue de Molsheim, Strasbourg Cedex 67082, France.

E-mail: erbs@transgene.fr

Received 22 July 2007; revised 5 September 2007; accepted 14 September 2007; published online 9 November 2007

vaccinia virus, which has been used safely as a smallpox vaccine during the end stage of the smallpox eradication program.^{18,19} MVA is largely nonpropagative in human and other mammalian cells²⁰ but infection with MVA results in the rapid replication of viral DNA, and therefore rapid amplification of the *FCU1* transgene. In addition there are now several vaccinia viral promoters with varying kinetics of activity and transcriptional strengths which are available to drive the transgene.²¹ Three vaccinia virus promoters were assessed for their ability to express the FCU1 fusion protein in the MVA context and the synthetic p11K7.5 promoter was found to drive production of the highest concentration of FCU1 *in vitro*. Intratumoral injections of MVA expressing *FCU1* into human tumors implanted subcutaneously into mice, with concomitant systemic administration of 5-FC, led to substantial delays in tumor growth and lower doses of the prodrug 5-FC were required to obtain a tumor growth control in comparison with Ad-FCU1. In addition we present data showing *in vivo* that 5-FC was converted to 5-FU in tumors by MVA-FCU1 and that 5-FU production was localized to the tumors. Replication competent vaccinia virus have previously been explored as vectors for suicide gene transfer to tumor cells²² but to our knowledge, this is the first report to assess the therapeutic efficacy of MVA expressing a suicide gene for cancer gene therapy and these results demonstrate that MVA-FCU1/5-FC provides a solution for generating therapeutic concentrations of 5-FU locally, while avoiding excessive toxicity in normal tissues.

Materials and methods

Cell culture

Primary chicken embryo fibroblasts (CEF) were prepared from chicken embryo obtained from fertilized eggs (Charles River SPAFAS, Germany) previously incubated 11 days at 37 °C in a humid atmosphere. Chicken embryos were dissected and treated with a solution of trypsin 2.5% (w/v). CEF cells were maintained in MBE supplemented with 10% fetal bovine serum. Human colon cancer cell lines SW480, WiDr and LoVo, human breast cancer cell lines MCF7, T-47D and MDA-MB-231, human pancreas cancer cell line Capan-2, human hepatocellular carcinoma cell line Hep G2 and human lung cancer cell line A549 were obtained from the ATCC (Rockville, MD). All human cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum.

Plasmid constructions, virus production and MVA transduction

The different MVA shuttle plasmids contain the early-late vaccinia promoters p7.5,²³ pH5R²⁴ or p11K7.5 (GenBank Accession Number CS054492, kindly provided by R Wittek, University of Lausanne) surrounded by the flanking sequences of the deletion III of the MVA (DelIII-R and DelIII-L sequences). The shuttle plasmids also contain the *E. coli* xanthine-guanine phosphoribosyl-

transferase gene (*GPT* gene) under the control of the pH5R vaccinia virus early-late promoter. Synthesis of xanthine-guanine phosphoribosyltransferase enables *GPT*⁺ recombinant MVA to form plaques in a selective medium containing mycophenolic acid, xanthine and hypoxanthine.²⁵ A restriction fragment *HindIII-KpnI* containing *FCU1* from plasmid pCI-neo*FCU1*¹⁴ was inserted downstream of the different vaccinia promoters. The resulting shuttle plasmids are described in Figure 1. In these constructions the selection marker *GPT* is placed between two homologous sequences in the same orientation (DelIII-L sequence). Generation of MVA was performed by infection of CEF with a subclone of MVA-null named MVAN33 isolated in our laboratory, followed by transfection of the different shuttle plasmids by CaCl₂ precipitation. Double recombination occurs between homologous regions (DelIII-R and DelIII-L) in the shuttle plasmid and the virus, resulting in the insertion of the gene cassette into the so-called deletion III of MVA. Recombinant MVA viruses are isolated by mycophenolic acid selection and multiple purification steps. The selection marker is easily eliminated by several passages without selection allowing the growth of *gpt*⁻ recombinant MVA obtained after intragenic homologous recombination between the two DelIII-L sequences flanking the *GPT* gene. The same methods were used to generate the MVAp11K7.5-GFP, which expresses *GFP* under the control of the p11K7.5 promoter. Virus structures were confirmed by multiple PCRs. Final recombinant MVA viruses were amplified in CEF and virus stocks were titrated on CEF by plaque assay. The control vector, MVA-null is defined as the MVAN33 without any inserted transgene.

The Ad-FCU1 vector is an E1/E3-deleted vector derived from the human adenovirus serotype 5 with an expression cassette in the E1 region containing the *FCU1* gene driven by the CMV immediate-early enhancer/promoter.¹⁴ The control vector Ad-null is similar except that it contains no transgene.¹⁴

To determine the *in vitro* transduction efficiency of MVA, cells were infected with MVAp11K7.5-GFP and 24 h later, single-cell suspensions were analyzed by flow cytometry using a FACScan instrument (Becton Dickinson, Le Pont de Claix, France).

Western blot analysis

WiDr tumor cells were infected with each MVA vector at a multiplicity of infection (MOI) of 0.01 and incubated for 24 h. Cell lysate proteins (30 µg) (determined using a Bio-Rad protein assay) were run on a 10% SDS-PAGE gel under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was incubated with rabbit anti-FCU1 peptide (codons 112–126) anti-serum at a 1:500 dilution, washed and incubated with secondary antibody coupled horseradish peroxidase (Amersham, Les Ulis, France). Signal detection was done by enhanced chemiluminescence (Amersham). The density of the bands was quantified using a densitometer (Imaging Densitometer, Bio-Rad, Marnes-la-Coquette, France).

Enzymatic assays

CDase activity, UPRTase activity and CDase-UPRTase activities in WiDr cells were determined using 5-FC (Toronto Research Chemicals Inc., North York, Canada) and 5-FU (Sigma, Saint Quentin Fallavia, France) as substrates. WiDr human tumor cells (5×10^6 cells) were infected with each MVA vector at a MOI of 0.01. Twenty four hours later, enzymatic assays were determined as previously described.¹⁴ 5-FC, 5-FU and 5-FUMP were

separated isocratically using HPLC (Hewlett Packard HP 1100 liquid chromatograph with UV detection at 260 nm and 280 nm). A Supelco supelcosil LC-18-S (5 μ m packing; 4.6×250 mm) column and a guard cartridge (10 $\times$ 3 mm; Varian, Les Ulis, France) were used with a flow rate of 1 ml min⁻¹. The mobile phase was 20 mM KH₂PO₄, 5 mM tetrabutylammoniumsulfate, 5% methanol adjusted to pH5 with potassium hydroxyde.

In vitro cell sensitivity to 5-FC

Human tumor cells were transduced by the respective recombinant MVA at a MOI of 0.01. A total of 2×10^5 transduced cells per well were incubated in six-well culture dishes in 2 ml of medium containing various concentrations of 5-FC. Cells were cultured at 37 °C for 6 days, and the viable cells were counted by trypan blue exclusion. Results are expressed as the ratio between the number of viable cells in plates containing the drugs versus the number of viable cells in the corresponding drug-free controls.

Analysis of the in vitro bystander effect

To determine whether transduced cells exposed to 5-FC mediated the production of 5-FU in the media to affect neighboring cells, WiDr cells, infected with the different vectors at a MOI of 0.01, were incubated in 12-well culture dishes (10⁶ cells per well). After 24 h, 1 mM 5-FC was added to the cultures. After 6, 24 and 48 h, the concentrations of 5-FC and 5-FU in the media were measured using HPLC. Media (50 μ l) were quenched with 1 ml of ethyl acetate/2-propanol/0.5 M acetic acid solution (84:15:1). The samples were vortexed and centrifuged. The organic supernatant was evaporated to dryness under a stream of nitrogen at 60 °C and reconstituted in 50 μ l of water and analyzed by HPLC as described above using a mobile phase of 50 mM phosphoric acid adjusted to pH 2.1 with ammonium hydroxide.

Figure 1 Generation of MVA expressing the *FCU1* gene and evaluation of *FCU1* protein expression. (a) MVA shuttle plasmids containing the *FCU1* gene. The *FCU1* gene is under the control of three different early-late promoters (p7.5, pH5R or p11K7.5). The *GPT* gene is driven by the vaccinia virus pH5R early-late promoter. DelIII-R and DelIII-L: flanking sequences surrounding deletion III of MVA. AmpR: β -lactamase coding region. Numbers refer to base pairs. (b) Western blot analysis. WiDr cells were infected with MVA at a MOI of 0.01 for 24 h. Protein was extracted and electrophoresed in a 10% SDS-PAGE gel. The expression level was analyzed by western blot using rabbit anti-*FCU1* peptide antiserum. Lane 1 (left to right), mock-infected WiDr cells; Lane 2, WiDr cells infected with MVA-null; Lane 3, WiDr cells infected with MVAp7.5-FCU1; Lane 4, WiDr cells infected with MVApH5R-FCU1; Lane 5, WiDr cells infected with MVAp11K7.5-FCU1. Molecular weight standards are shown in kDa on the left. The presence of *FCU1* (M_r 42000) is indicated. (c) Relative amount of the immunoreactive *FCU1* protein expression. The relative density at each point was calculated by dividing that value by the density of WiDr cells infected with MVAp11K7.5-FCU1 (100%).

Animal experiments

Female Naval Medical Research Institute (NMRI) nude mice were obtained from Charles River Laboratories (Saint Aubin-les-Elbeuf, France). Animals used in the studies were uniform in age (6 weeks) and body weights ranged from 23–26 g. NMRI nude mice were injected subcutaneously (s.c.) into the flank with 5×10^6 LoVo cells. When tumors reached a diameter of 60–80 mm³ (this size of tumor contains approximately 5×10^7 to 1×10^8 cells), the mice were randomized in a blinded manner and treated with the indicated vectors for the *in vivo* experiments.

Determination of tumor and blood concentration of 5-FC and converted 5-FU

Using human tumor LoVo cells implanted s.c. in nude mice, control vehicle (100 µl PBS) or 5.10^6 PFU of MVAp11K7.5-FCU1 (resuspended in 100 µl PBS) were intratumorally injected on day 0. The prodrug 5-FC was given by oral gavage at 100 mg kg⁻¹ per day (0.5 ml 5-FC 0.5% in water) twice daily for 2 weeks. Animals were killed 1 h post-5-FC gavage on days 1, 3, 6, 8, 10 and 14, and blood samples and tumors were collected. Plasma was separated by centrifugation from blood collected via the retro-orbital sinus in heparinized tubes. Tumors were homogenized using a Polytron homogenator. Tumor or plasma samples were quenched with 1 ml of ethyl acetate/2-propanol/0.5 M acetic acid solution (84:15:1). The organic supernatant was reconstituted in 50 µl of water and analyzed by HPLC as described above using 50 mM phosphoric acid adjusted to pH 2.1 as mobile phase.

Antitumor activity of MVA-FCU1 versus Ad-FCU1

Human tumors were treated with the MVAp11K7.5-FCU1 or Ad-FCU1 vectors followed by *per os* 5-FC administration at different concentration. Animals were treated intratumorally with the indicated vectors at dose of 5.10^8 IU (Ad-null, Ad-FCU1) or 5.10^6 PFU (MVA-null, MVAp11K7.5-FCU1). The vectors or the control vehicle (PBS) were directly injected into the tumor at days 11, 13 and 15 after tumor implantation. From day 11 on, 5-FC was given by oral gavage at 100 mg kg⁻¹ per day (0.5 ml 5-FC 0.5% in water), 250 mg kg⁻¹ per day (0.6 ml 5-FC 1%) or 500 mg kg⁻¹ per day (0.6 ml 5-FC 2%) twice a day for 2 weeks. Tumor volumes were calculated in mm³ using the formula $(\pi/6)(\text{length} \times \text{width}^2)$.

Statistical analysis

Statistical analyses were performed using the nonparametric Mann–Whitney *U*-test and STATISTICA 7.1 software (StatSoft Inc.). A $P < 0.05$ was considered to be statistically significant.

Results

In vitro infection of human tumor cells by MVA

Nine different human cancer cell lines, representing five different human tumor types, were tested for their

Table 1 Susceptibility of human tumor cell lines to MVA infection

Cell line	MOI	Transduction efficiency ^a
A549	0.01	18.7 ± 0.1
	0.1	76.7 ± 0.3
MCF7	0.01	12.7 ± 0.5
	0.1	63.2 ± 1.2
T-47D	0.01	11.8 ± 0.1
	0.1	58.2 ± 0.2
MDA-MB-231	0.01	17.5 ± 0.3
	0.1	75.9 ± 0.4
LoVo	0.01	5.2 ± 0.3
	0.1	25.7 ± 1.1
SW480	0.01	9.5 ± 0.1
	0.1	57.1 ± 0.5
WiDr	0.01	3.8 ± 0.1
	0.1	27.5 ± 0.4
Capan-2	0.01	8.1 ± 0.2
	0.1	44.3 ± 0.5
Hep G2	0.01	12.2 ± 0.2
	0.1	52.3 ± 0.7

Abbreviation: MOI, multiplicity of infection; MVA, modified vaccinia virus Ankara.

^aTumor cells were infected at the indicated MOI with MVAp11K7.5-GFP and the percentage of GFP-positive cells was determined by fluorocytometry at 24 h post infection from scoring a minimum of 10 000 cells. The results were obtained from three separated experiments ± s.d.

susceptibility to infection *in vitro* with an MVA expressing GFP marker protein under the control of the p11K7.5 promoter (MVA p11K7.5-GFP). The cell lines were infected with MVA p11K7.5-GFP at an MOI of 0.01 and 0.1 PFU and the transduction efficiency was assessed by flow cytometry 24 h post infection. The results are summarized in Table 1. The 50% transduction efficiency of this vector in human cancer cell lines varied, but all these cell lines showed 25–75% transduction efficiency at an MOI of 0.1. The observed infection frequency which was slightly higher than expected can be explained by the previous observation²⁶ that one plaque forming unit of MVA is often an aggregate of several viral particles. The transduction efficiencies of human tumor cells by MVA at an MOI of 0.1 were comparable to those obtained with an E1/E3 deleted, serotype 5 adenovirus vector at an MOI of 1.¹⁴

Expression of FCU1 fusion protein in WiDr cells infected with MVA-FCU1

Western blot analysis of the FCU1 protein, using FCU1 specific rabbit antiserum, was done to compare the levels of FCU1 produced by the three vaccinia virus promoters. An FCU1 specific signal was observed in cellular extracts isolated from WiDr cells infected by MVA-FCU1

(Figure 1b, Lanes 3–5). The apparent molecular size of FCU1 protein was 42 kDa, in agreement with the expected size of an *S. cerevisiae* FCY1:FUR1 fusion protein. The relative density of the FCU1 protein level indicated a 2.7-fold increase in WiDr cells after infection with MVApH5R-FCU1 (Figure 1c, Lane 4), compared to MVAp7.5-FCU1-infected cells (Lane 3). The level of FCU1 protein was elevated 6.7 and 2.5-fold in cells infected with MVAp11K7.5-FCU1 (Lane 5) compared to MVAp7.5-FCU1-infected cells (Lane 3) and MVApH5R-FCU1-infected cells (Lane 4), respectively. These results show that the p11K7.5 promoter led to the highest level of FCU1 expression.

Functionality of the FCU1 fusion gene

In order to confirm the strongest promoter driving expression of FCU1 protein and to determine whether FCU1 is functional when produced under the direction of each of the three promoters, WiDr human tumor cells were infected with each vector at an MOI of 0.01 and the specific CDase, UPRTase and CDase/UPRTase enzymatic activities were then determined 24 h later, as described in Materials and methods. As shown in Table 2, elevated CDase activity was found in cells infected with MVAp11K7.5-FCU1, while no CDase activity was detectable in mock-infected cells or in MVA-null-infected cells. No endogenous UPRTase activity was detected in uninfected WiDr cells (Table 2). Consistent with the western blot analysis (Figure 1b), higher UPRTase activity was recovered using the p11K7.5 construct. Analysis of the direct conversion of 5-FC to 5-FUMP,

indicative of the existence of a combined CDase-UPRTase activity, confirmed that *FCU1* does encode a bifunctional enzyme (Table 2). Moreover, this combined enzymatic activity was found to be 5.8- and 2.3-fold higher in cells infected with MVAp11K7.5-FCU1 than in cells infected with MVAp7.5-FCU1 and MVApH5R-FCU1, respectively. Taken together, these *in vitro* enzymatic assays confirm that the p11K7.5 promoter is the most active promoter driving the FCU1 bifunctional enzyme.

Enhanced sensitivity of human tumor cells to 5-FC after infection with MVA-FCU1 via the 5-FC-mediated bystander effect

To test whether MVA-mediated *FCU1* gene expression in the cells conferred sensitivity to 5-FC, nine different human tumor cell lines were infected with MVA-null, MVAp7.5-FCU1, MVApH5R-FCU1 or MVAp11K7.5-FCU1 at a MOI of 0.01 and then exposed to 5-FC at various concentrations for six subsequent days. As shown in Figure 2, all cell lines infected with the MVA-FCU1 became sensitive to 5-FC. Consistent with the results from the enzymatic analyses (Table 2), infection with MVAp11K7.5-FCU1 confers to the cells the highest sensitivity to 5-FC when compared with the MVAp7.5-FCU1- or MVApH5R-FCU1-infected cells. For example, infection of MDA-MB-231 and Capan-2 cells with MVAp11K7.5-FCU1 followed by 5-FC treatment resulted in a 10- and 100-fold shift in IC_{50} compared with MVApH5R-FCU1 and MVAp7.5-FCU1 infections, respectively (Figure 2). As expected, infection with MVA-null did not modify the sensitivity of the cells to 5-FC. Given the relatively weak efficiency of infection at 0.01 PFU/cell (Table 1), these results suggest that a bystander effect occurs under these treatment conditions. To investigate this 5-FC-mediated bystander effect, analysis of the cell culture supernatant by HPLC revealed a progressive increase in the amount of 5-FU in the extracellular milieu of WiDr cells transduced with MVA-FCU1 at an MOI of 0.01 and incubated with 1 mM 5-FC (Figure 3a). This result indicates that when only 4% of WiDr cells were transduced (Table 1), 5-FC was efficiently converted by MVA-FCU1 infected cells and that 5-FU was freely diffusible across cell membranes. As expected, MVAp11K7.5-FCU1-infected cells were much more effective at transforming 5-FC into 5-FU, with a mean conversion ratio of 72% after 2 days of incubation.

In vivo 5-FC/5-FU conversion in human tumors by MVAp11K7.5-FCU1

LoVo tumors were established in nude mice and the concentrations of 5-FU in tumor tissue at different days post infection were determined after a single intratumoral

Table 2 Specific CDase, UPRTase and CDase/UPRTase activities in WiDr cell line

Vector	CDase 5-FC→5-FU	UPRTase 5-FU→5-FUMP	CDase/UPRTase 5-FC→5-FUMP
Mock	ND	ND	ND
MVA-null	ND	ND	ND
MVAp7.5-FCU1	994 ± 105	282 ± 33	140 ± 18
MVApH5R-FCU1	3092 ± 218	808 ± 53	360 ± 43
MVAp11K7.5-FCU1	6160 ± 326	1605 ± 89	811 ± 73

Abbreviations: CDase, cytosine deaminase; ND, not detectable; UPRTase, uracil phosphoribosyltransferase.

CDase, UPRTase and CDase/UPRTase activities are expressed as the number of pmoles of 5-FC deaminated per min per mg of protein, the number of pmoles of 5-FU phosphorylated per min per mg of protein and the number of pmoles of 5-FUMP produced per min per mg of protein when using 5-FC as substrate, respectively.

The indicated enzymatic activities were measured as described in Materials and methods. Each value represents the average of three independent experiments ± s.d.

Figure 2 *In vitro* sensitivities of MVA-transduced human tumor cells to 5-FC. Cells transduced at a MOI of 0.01 with the indicated vectors were exposed to various concentrations of 5-FC and cell survival was measured 6 days later as described in Materials and methods. Results are expressed as the percentage of surviving cells in presence and absence of the drug. Values are represented as mean ± s.d. of four individual determinations.

Figure 3 5-FU generated *in vitro* and *in vivo* by MVA-FCU1 prodrug system. **(a)** Conversion of 5-FC to 5-FU and release of 5-FU in the cell culture supernatant. WiDr cells were infected with the indicated vectors at a MOI of 0.01 and then incubated with 1 mM 5-FC. The relative concentrations of 5-FC and 5-FU in the media were measured by HPLC, 6, 24 and 48 h later. The data are expressed as the percentage of 5-FU in the media relative to the total amount of 5-FC + 5-FU. Each data point represents the mean $\pm$ s.d. of triplicate determinations. **(b)** Intratumoral concentration of 5-FU in human tumors. Tumors were established by s.c. implantation of 5×10^6 LoVo cells into nude mice. When tumors had reached 60–80 mm³, the animals were intratumorally inoculated with MVAp11K7.5-FCU1 (5.10^6 PFU) and treated for 2 weeks with *per os* administrations of 5-FC. At 1, 3, 6, 8, 10 and 14 days after viral injection, tumor tissues were collected 1 h after a single dose of 5-FC (100 mg kg^{-1}) and concentration of 5-FU in the tumor was measured by HPLC as described in Materials and methods. Three mice were used for each group.

injection of MVA p11K7.5-FCU1 (Figure 3b). The highest levels of 5-FU were detected in tumor tissues 6 days post infection. At 14 days post infection, 5-FU was still detected in the tumor tissues. In mice treated with 5-FC only, the concentration of 5-FU in tumors was undetectable (Figure 3b) indicating that no endogenous conversion of 5-FC to 5-FU occurs in the tumors. The concentrations of 5-FU in serum in mice receiving 5-FC, with and without MVA-FCU1 in tumors, were also determined. No 5-FU was detected in serum of the group

treated with 5-FC alone. Similarly, 5-FU could not be detected in serum of mice treated with MVA-FCU1/5-FC (data not shown) suggesting that 5-FU does not diffuse from the tumor tissues. In contrast, in the mice treated with a single injection of 5-FU at the maximum tolerated dose (10 mg kg^{-1} administered intraperitoneally), the blood concentration of 5-FU reached a maximum of $5 \mu\text{g ml}^{-1}$ 5 min after injection of 5-FU and the highest level of 5-FU in tumor tissues was detected 10 min post injection with a concentration of $0.6\text{--}0.7 \text{ ng mg}^{-1}$ of tumor. When a single intraperitoneal injection of 5-FU at 200 mg kg^{-1} (20-fold the maximum tolerated dose) was given, the 5-FU level reached a maximum of $90 \mu\text{g ml}^{-1}$ of serum and 10 ng mg^{-1} of tumor 10 min post-5-FU administration. One hour after 5-FU injection, 5-FU became undetectable in tumors. The intratumoral 5-FU level resulting from MVA-FCU1 was equivalent to the maximum level attained with systematically administered 5-FU at 20-fold the maximum tolerated dose.

In vivo efficacy of MVA-mediated transfer of FCU1

To examine the therapeutic effects of MVA-FCU1 *in vivo*, nude mice bearing established LoVo colon tumors were injected intratumorally with MVA-null, MVAp11K7.5-FCU1 or mock (vehicle alone) with a concomitant *per os* administration of either 5-FC (at 1000, 500 or 200 mg kg^{-1} per day) or water for 14 days. As shown in Figure 4a, administration of MVAp11K7.5-FCU1/5-FC resulted in a statistically significant suppression of tumor growth whatever the dose of 5-FC, whereas no modification in tumor growth was observed in the control groups. At 5 weeks after delivery of MVAp11K7.5-FCU1/5-FC, the LoVo tumors were 75% smaller, respectively, compared to the controls (all controls averaged; Figure 4a). In parallel, control experiments were also performed to determine the *in vivo* antitumor effect of 5-FU in nontransduced LoVo tumor cells. Despite the administration of doses of 5-FU that were at the maximum tolerated concentrations (i.p. injection of 10 mg of 5-FU per kg twice daily during 2 weeks), no statistically significant inhibition of tumor growth was observed (data not shown). These results indicate that the significant antitumor effect was due to the local production of high concentrations of 5-FC derivatives via the *FCU1* gene product expressed from the p11K7.5 promoter within the MVA vector. MVAp11K7.5-FCU1/5-FC was also compared to replication-deficient adenovirus expressing *FCU1*. Using adenovirus, administration of *FCU1* with a concomitant *per os* administration of 5-FC at 1000 mg kg^{-1} per day resulted in a statistically significant suppression of tumor growth compared to the control groups, whereas no modification in tumor growth was observed with administration of 5-FC at 500 and 200 mg kg^{-1} per day (Figure 4b). The data in Figure 4 indicate that the transfer of the *FCU1* gene to tumors is much more efficient using the MVA system than the adenovirus system previously described.¹⁴ MVAp11K7.5-FCU1 allows the same therapeutic effect at one-fifth of the daily dose of 5-FC in comparison to Ad-FCU1.

Figure 4 Antitumor effect of the MVAp11K7.5-FCU1/5-FC (a) and Ad-FCU1/5-FC (b) therapies on established human LoVo colon carcinoma ($n=14$ per group). 5×10^6 LoVo cells implanted s.c. into nude mice were injected at days 11, 13 and 15 with the vehicle alone or with either 5×10^6 PFU of MVA (a) or 5×10^8 IU of Adenovirus (b). The animals were then treated twice daily with *per os* administrations of 5-FC for two weeks at 1000, 500 or 200 mg kg⁻¹ per day. In MVA groups, differences in tumor sizes between the group treated with MVAp11K7.5-FCU1 + 5-FC and the other groups was shown to be statistically significant ($P<0.01$), whatever the dose of 5-FC. In groups treated with adenovirus, differences in tumor sizes between the group treated with Ad-FCU1 + 5-FC and the other groups was shown to be statistically significant ($P<0.01$) only with 5-FC at 1000 mg kg⁻¹ per day.

Discussion

MVA, a member of the poxvirus family, was derived from the vaccinia virus strain Ankara by over 570 serial passages in CEF cells.¹⁸ The resulting MVA strain was unable to productively grow in cell cultures of human origin.²⁰ MVA vaccine was used during the end stage of the smallpox eradication program in Germany and Turkey in more than 120 000 humans without documentation of any of the complications associated with other vaccinia viruses.¹⁹ Furthermore recent preclinical studies have shown that MVA is avirulent upon inoculation of various animals.^{27,28} Because of its high degree of attenuation, MVA has appeared as an attractive alternative to standard vaccinia strains for the development of viral vectors to be used in vaccination or immunotherapy.²⁹ Like other attenuated poxvirus vectors used as recombinant vaccines (ALVAC and NYVAC), MVA will not propagate in most mammalian cells. However, unlike ALVAC³⁰ and NYVAC,³¹ MVA infection results in the replication of viral DNA.²⁰ This, in turn, results in the high level production of recombinant proteins.²⁰ MVA also has many other advantages as a live viral vaccine vector: (1) large foreign gene capacity; (2) long-term stability in frozen or lyophilized state; (3) replication exclusively in the cytoplasm eliminating any risk of integration.

In this report, we assessed the ability of MVA to transfer the *FCU1* genetic sequence to tumor cells *in vitro* and *in vivo*, and compared it to the previously reported Ad-FCU1.¹⁴ We first tested altogether nine human cancer

cell lines representing five different tissues for transduction efficiency. Conditions have been established for each vector where some preclinical efficacy has been demonstrated. Our findings demonstrate that an MVA vector can efficiently transduce human tumor cells *in vitro*, allowing 25–75% gene transfer efficiency. Similar gene transfer efficiencies were obtained with an E1/E3 deleted adenovirus vector.¹⁴ It should be emphasized that it would be nonproductive to compare poxvirus- and adenovirus vectors directly because their titrating methods are different (technique of titration, target cell line, transduction volume, time of incubation) and therefore the real concentrations of biologically active virus may not be equal.

We have described a comparison of the efficacy of MVA vectors expressing *FCU1* under the control of three different promoters. *In vitro* experiments unambiguously showed that the p11K7.5 promoter constitutively drives the highest level of *FCU1* as assessed by protein levels and enzymatic assays. Similarly, pH5R promoter strength was intermediate between that of p11K7.5 and p7.5. This synthetic 110-bp p11K7.5 promoter, which consists in a fusion between the p11K late promoter and the p7.5 early-late promoter, has been successfully used in our laboratory for the expression of GFP and other foreign proteins by MVA and vaccinia virus.

Our results indicated that MVA-FCU1 + 5-FC generates a very strong killing effect. The amount of 5-FU produced by recombinant viruses in the media essentially eradicated >95% of tumor cells *in vitro*. We also confirmed that 5-FU diffuses in and out of cells and does

not require cell-to-cell contact for bystander activity. This bystander effect should enhance the antitumor efficacy of the suicide gene therapy approach by eliminating uninfected tumor cells.

Early work with bacterial CDase in combination with 5-FC was promising; however, later studies revealed that due to poor catalytic efficiency this system is likely to have limited clinical applicability.^{32,33} Recent studies^{34,35} have demonstrated that *S. cerevisiae* CDase deaminates 5-FC more efficiently than *E. coli* CDase, and that human and murine cancer cells transduced with the yeast *FCY1* gene were significantly more sensitive *in vitro* and *in vivo* to 5-FC than tumor cells transduced with the bacterial *codA* gene. This was attributed to the more favorable K_m and V_{max} of yeast CDase for 5-FC, which was 22-fold lower and 6-fold higher than those of bacterial CDase, respectively.^{34,35} In our previous report,¹⁴ we also noted an unexpected increase in the deaminase activity of the fusion protein FCU1 which was over 100-fold higher than the native CDase. Similarly, a study recently reported an increase in the catalytic activity of the *S. cerevisiae* CDase when expressed as a fusion protein with *Haemophilus influenzae* UPRTase.³⁶ This increased CDase activity is a consequence of the enhanced thermal stability of the fusion protein compared to native CDase allowing it to function for a greater period of time.³⁶

In this study, we have evaluated the conversion of 5-FC to 5-FU in tumors after a single intratumoral injection of MVA p11K7.5-FCU1 in combination with a *per os* regimen of 5-FC. After a single dose of 100 mg kg⁻¹ 5-FC, the converted 5-FU in tumors reached the highest level (5.5 ng mg⁻¹ of tumor) 3–8 days post infection. At 14 days post infection, 5-FU was still detected in the tumor tissues indicating the persistence of gene expression under the control of the p11K7.5 promoter and/or the high stability of FCU1 protein in the tumor mass. In our study, the intratumoral 5-FU level resulting from MVA-FCU1 was equivalent to the maximum level attained with systematically administered 5-FU at 20-fold the maximum tolerated dose. In addition we showed that after the direct production of 5-FU at the tumor site using MVA-FCU1, no 5-FU could be detected in serum resulting in tumor cell death without systemic toxicity. This high intratumoral concentration of 5-FU and its undetectable level in serum could be attributable to the retention ('trapping') of 5-FU in the tumor. Such a 'trapping' of 5-FU within tumor cells has been noted in a number of previous studies of 5-FU therapy^{37,38} and has been rationalized on the basis that the 5-FU half-life is significantly longer in tumors than in blood.^{37,39}

We compared the efficiency of *FCU1* gene transfer by MVA vector and nonreplicative-adenovirus, both of which are unable to proliferate in human tumors. Our finding demonstrates that both types of vectors transduce proliferating tumor cells *in vivo* with comparable efficiency. After a single intratumoral injection of 5.10⁸ IU of adenovirus expressing GFP¹⁴ or 5.10⁶ PFU of MVA-GFP, only a few percent (1–5%) of tumor cells are GFP⁺ and these cells were localized along the needle track (data not shown). At these doses of MVA or adenovirus, when

high concentration of 5-FC were used (1000 mg kg⁻¹ per day), both MVA-FCU1 and Ad-FCU1 vectors suppressed human colon tumor growth. These Ad-FCU1 results were consistent with our previously reported experiments.¹⁴ At relatively lower 5-FC doses (500 and 200 mg kg⁻¹ per day) MVA-FCU1 could also suppress tumor growth, whereas no modification of tumor growth was observed using Ad-FCU1. These results suggest an increased transgene expression in MVA context in comparison to Ad-FCU1 and further investigations are needed to determine level and persistence of gene expression after direct injection of MVA in solid human tumors. 5-FC is used clinically for antifungal therapy and in this context is typically administered daily for 6 weeks. Nontoxic systemic serum levels of 50–100 µg ml⁻¹ are achievable with oral administration of 100–200 mg kg⁻¹ per day in four divided doses.⁴⁰ Our findings, of effective cell killing with tolerable equivalent human doses of 5-FC suggest significant advantages in using MVA-mediated transfer of FCU1 for molecular therapy since in previous investigations involving 5-FC/CDase a dose of at least 500 mg kg⁻¹ 5-FC was required for therapeutic effects in tumor-bearing animals.^{2–7,34–36,41} Attempts to reduce the dose of 5-FC to less than 500 mg kg⁻¹ resulted in no effect on tumor growth.⁴²

In summary, this study represents, to our knowledge, the first study to use MVA vector as gene transfer vehicle for suicide gene therapy. It demonstrates the efficacy of intratumoral 5-FU produced via intratumoral injection of MVA-FCU1 and a regimen of systemic 5-FC at well-tolerated doses. The results of the present study also demonstrate the superior efficacy of MVA in comparison to the traditionally used adenovirus in delivery of a suicide gene in human tumor cells.

We believe that MVA-FCU1 is highly promising for clinical development for the local control of cancers, such as colorectal liver metastases, hepatocarcinoma, glioma or prostate cancer and could also be combined with other therapies, such as radiotherapy, thus exploiting 5-FU radiosensitizing potential.⁴¹

Acknowledgements

We thank R Wittek (University of Lausanne, Switzerland) for providing the synthetic vaccinia p11K7.5 promoter. We thank Y Schlesinger and D Villev for their technical assistance. We would like to acknowledge the help and scientific criticisms of B. Acres during the preparation of this manuscript.

References

- 1 Mullen CA, Kilstrup M, Blaese RM. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc Natl Acad Sci USA* 1992; **89**: 33–37.
- 2 Hirschowitz EA, Ohwada A, Pascal WR, Russi TJ, Crystal RG. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon

- carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther* 1995; **6**: 1055–1063.
- 3 Kanai F, Lan KH, Shiratori Y, Tanaka T, Ohashi M, Okudaira T et al. In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res* 1997; **57**: 461–465.
- 4 Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994; **91**: 8302–8306.
- 5 Miller CR, Williams CR, Buchsbaum DJ, Gillespie GY. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. *Cancer Res* 2002; **62**: 773–780.
- 6 Lee CH, Wu CL, Shiau AL. Hypoxia-induced cytosine deaminase gene expression for cancer therapy. *Hum Gene Ther* 2007; **18**: 27–38.
- 7 Guffey MB, Parker JN, Luckett Jr WS, Gillespie GY, Meleth S, Whitley RJ et al. Engineered herpes simplex virus expressing bacterial cytosine deaminase for experimental therapy of brain tumors. *Cancer Gene Ther* 2007; **14**: 45–56.
- 8 Erbs P, Exinger F, Jund R. Characterization of the *Saccharomyces cerevisiae* FCY1 gene encoding cytosine deaminase and its homologue FCA1 of *Candida albicans*. *Curr Genet* 1997; **31**: 1–6.
- 9 Danielsen S, Kilstrop M, Barilla K, Jochimsen B, Neuhaard J. Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. *Mol Microbiol* 1992; **6**: 1335–1344.
- 10 Koechlin BA, Rubio F, Palmer S, Gabriel T, Duschinsky R. The metabolism of 5-fluorocytosine-2-14-C and of cytosine-14-C in the rat and the disposition of 5-fluorocytosine-2-14-C in man. *Biochem Pharmacol* 1966; **15**: 435–446.
- 11 Andersen PS, Smith JM, Mygind B. Characterization of the *upp* gene encoding uracil phosphoribosyltransferase of *Escherichia coli* K12. *Eur J Biochem* 1992; **204**: 51–56.
- 12 Kern L, de Montigny J, Jund R, Lacroute F. The FUR1 gene of *Saccharomyces cerevisiae*: cloning, structure and expression of wild-type and mutant alleles. *Gene* 1990; **88**: 149–157.
- 13 Kanai F, Kawakami T, Hamada H, Sadata A, Yoshida Y, Tanaka T et al. Adenovirus-mediated transduction of *Escherichia coli* uracil phosphoribosyltransferase gene sensitizes cancer cells to low concentrations of 5-fluorouracil. *Cancer Res* 1998; **58**: 1946–1951.
- 14 Erbs P, Regulier E, Kintz J, Leroy P, Poitevin Y, Exinger F et al. In vivo cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. *Cancer Res* 2000; **60**: 3813–3822.
- 15 Trinh QT, Austin EA, Murray DM, Knick VC, Huber BE. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. *Cancer Res* 1995; **55**: 4808–4812.
- 16 Mesnil M, Piccoli C, Tiraby G, Willecke K, Yamasaki H. Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc Natl Acad Sci USA* 1996; **93**: 1831–1835.
- 17 Matono S, Tanaka T, Sueyoshi S, Yamana H, Fujita H, Shirouzu K. Bystander effect in suicide gene therapy is directly proportional to the degree of gap junctional intercellular communication in esophageal cancer. *Int J Oncol* 2003; **23**: 1309–1315.
- 18 Mayr A, Stickl H, Muller HK, Danner K, Singer H. The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's translation). *Zentralbl Bakteriol [B]* 1978; **167**: 375–390 (in German).
- 19 Stickl H, Hochstein-Mintzel V, Mayr A, Huber HC, Schafer H, Holzner A. MVA vaccination against smallpox: clinical tests with an attenuated live vaccinia virus strain (MVA) (author's translation). *Dtsch Med Wochenschr* 1974; **99**: 2386–2392 (in German).
- 20 Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc Natl Acad Sci USA* 1992; **89**: 10847–10851.
- 21 Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* 1997; **23**: 1094–1097.
- 22 Puhlmann M, Gnant M, Brown CK, Alexander HR, Bartlett DL. Thymidine kinase-deleted vaccinia virus expressing purine nucleoside phosphorylase as a vector for tumor-directed gene therapy. *Hum Gene Ther* 1999; **10**: 649–657.
- 23 Cochran MA, Puckett C, Moss B. In vitro mutagenesis of the promoter region of vaccinia virus gene: evidence for tandem early and late regulatory signals. *J Virol* 1985; **54**: 30–37.
- 24 Rosel JL, Earl PL, Weir JP, Moss B. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment. *J Virol* 1986; **60**: 436–439.
- 25 Falkner F, Moss B. *Escherichia coli* gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J Virol* 1988; **62**: 1849–1854.
- 26 Spohner D, Drillien R, Proamer F, Houssais-Pecher C, Zanta MA, Geist M et al. Enveloped virus is the major virus form produced during productive infection with the modified vaccinia virus Ankara strain. *Virology* 2000; **273**: 9–15.
- 27 Ramirez JC, Gherardi MM, Esteban M. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. *J Virol* 2000; **74**: 923–933.
- 28 Stittelaar KJ, Kuiken T, de Swart RL, van Amerongen G, Vos HW, Niesters HG et al. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine* 2001; **19**: 3700–3709.
- 29 Sutter G, Staib C. Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery. *Curr Drug Targets Infect Disord* 2003; **3**: 263–271.
- 30 Taylor J, Weinberg R, Tartaglia J, Richardson C, Alkhatib G, Briedis D et al. Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. *Virology* 1992; **187**: 321–328.
- 31 Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI et al. NYVAC: a highly attenuated strain of vaccinia virus. *Virology* 1992; **188**: 217–232.
- 32 Pederson LC, Buchsbaum DJ, Vickers SM, Kancharla SR, Mayo MS, Curiel DT et al. Molecular chemotherapy combined with radiation therapy enhances killing of cholangiocarcinoma cells in vitro and in vivo. *Cancer Res* 1997; **57**: 4325–4332.
- 33 Lambin P, Nuyts S, Landuyt W, Theys J, De Bruijn E, Anne J et al. The potential therapeutic gain of radiation-associated gene therapy with the suicide gene cytosine deaminase. *Int J Radiat Biol* 2000; **76**: 285–293.

- 34 Kievit E, Bershad E, Ng E, Sethna P, Dev I, Lawrence TS *et al*. Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. *Cancer Res* 1999; **59**: 1417–1421.
- 35 Hamstra DA, Rice DJ, Fahmy S, Ross BD, Rehemtulla A. Enzyme/prodrug therapy for head and neck cancer using a catalytically superior cytosine deaminase. *Hum Gene Ther* 1999; **10**: 1993–2003.
- 36 Hamstra DA, Lee KC, Tychewicz JM, Schepkin VD, Moffat BA, Chen M *et al*. The use of ¹⁹F spectroscopy and diffusion-weighted MRI to evaluate differences in gene-dependent enzyme prodrug therapies. *Mol Ther* 2004; **10**: 916–928.
- 37 Wolf W, Presant CA, Servis KL, el-Tahtawy A, Albright MJ, Barker PB *et al*. Tumor trapping of 5-fluorouracil: *in vivo* ¹⁹F NMR spectroscopic pharmacokinetics in tumor-bearing humans and rabbits. *Proc Natl Acad Sci USA* 1990; **87**: 492–496.
- 38 Presant CA, Wolf W, Waluch V, Wiseman C, Kennedy P, Blayney D *et al*. Association of intratumoral pharmacokinetics of fluorouracil with clinical response. *Lancet* 1994; **343**: 1184–1187.
- 39 Presant CA, Jacobson J, Wolf W, Waluch V, Weitz IC, Macdonald JS. Does leucovorin alter the intratumoral pharmacokinetics of 5-fluorouracil (5-FU)? A Southwest Oncology Group study. *Invest New Drugs* 2002; **20**: 369–376.
- 40 Edwards Jr JE, Bodey GP, Bowden RA, Buchner T, de Pauw BE, Filler SG *et al*. International conference for the development of a consensus on the management and prevention of severe candidal infections. *Clin Infect Dis* 1997; **25**: 43–59.
- 41 Kievit E, Nyati MK, Ng E, Stegman LD, Parsels J, Ross BD *et al*. Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. *Cancer Res* 2000; **60**: 6649–6655.
- 42 Kambara H, Tamiya T, Ono Y, Ohtsuka S, Terada K, Adachi Y *et al*. Combined radiation and gene therapy for brain tumors with adenovirus-mediated transfer of cytosine deaminase and uracil phosphoribosyltransferase genes. *Cancer Gene Ther* 2002; **9**: 840–845.