THE JOURNAL OF GENE MEDICINE
J Gene Med 2015; **17**: 132–139.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/jgm.2831

RESEARCH ARTICLE

Redesigned *Escherichia coli* cytosine deaminase: a new facet of suicide gene therapy

Asif Raza[1]
V. Kohila[2]
Siddhartha Sankar Ghosh[1,3]*

[1]*Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, Assam, India*

[2]*Department of Biotechnology, National Institute of Technology, Warangal, India*

[3]*Centre for Nanotechnology, Indian Institute of Technology Guwahati, Guwahati, Assam, India*

*Correspondence to: S. Sankar Ghosh, Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati-39, Assam, India.
E-mail: sghosh@iitg ernet in

Received: 9 March 2015
Accepted: 12 May 2015

Abstract

Background The *Escherichia coli* cytosine deaminase (CD)/5-fluorocytosine (5-FC) approach emerges as a potential aid for suicide gene therapy in the field of modern cancer treatment. However, the poor binding affinity of CD towards 5-FC compared to the natural substrate cytosine limits its application for successful suicide gene therapy. Redesigning a bacterial mutant CD with site-directed mutagenesis showed higher potency compare to wild-type CD (wtCD) *in vitro*. In the present study, we conducted a comparative analysis of F186W mutant and wtCD in a human lung cancer cell line (A549).

Methods and Results A comparative investigation was initiated with cell viability analyses by MTT and trypan blue dye exclusion assays on A549 cells transfected with wtCD and F186W genes. The mode of cell death was confirmed by acridine Orange/ethidium Bromide dual staining. Furthermore, flow cytometric assessments were performed by cell cycle analysis and caspase 3 assay. The experimental results showed a drug dependent decrease in cell viability; interestingly, mutant (F186W) reached IC50 at a much lower concentration of prodrug (5-FC) than wtCD. Cell cycle analysis showed that G1 arrest of a larger population of 5-FC treated F186W transfected cells, in contrast to that of wtCD under similar conditions. The caspase 3 assay revealed progression and execution of apoptosis.

Conclusions We report a novel bacterial CD mutant that provided a superior alternate to the wtCD suicide gene. The F186W mutant required a much lower dose of 5-FC to reach its IC_{50}, thus minimizing the systemic side effects of large doses of 5-FC as required for wtCD.

Keywords cytosine deaminase; mutant; site-directed mutagenesis; suicide gene therapy

Introduction

Gene therapy currently has a great impact on the treatment options of various diseases, including cancer. As suicide gene therapy has entered various clinical trials, the factors limiting its potency cannot be overlooked. Gene-directed enzyme prodrug therapy (GDEPT) based on the principles of suicide gene therapy incorporates the intratumoral delivery of genes encoding enzymes, which activates prodrug [1]. GDEPT has potential to overcome the limitations of conventional cancer therapeutics because the former has the advantage of bystander effect, as well as targeted therapy [2–4]. Two combinations of

enzymes and prodrugs have been widely studied: the *Escherichia coli* cytosine deaminase (CD; EC 3.5.4.1) with antifungal drug 5-fluorocytosine (CD/5-FC) and the herpes simplex virus thymidine kinase (HSVtk) with the anti-herpetic ganciclovir (GCV) [5–10]. HSVtk/GCV therapy exhibits more cytotoxicity towards cancer cells as a result of its gap junction-dependent bystander effect [5]. Because the triphosphate GCV cannot diffuse through the cell membrane, its efficacy is limited towards gap junction deficient cancer cells [11]. However, the CD/5-FC system does not rely on gap junction because the toxic product 5-fluorouracil (5-FU) can freely diffuse across the cell membrane, overcoming the limitations of the HSVtk/GCV system [12].

The CD enzyme catalyzes the hydrolytic deamination of cytosine into uracil. It can also convert the inert prodrug 5-FC into highly toxic chemotherapeutic drug 5-FU, which is then further converted into potent antimetabolites (5-FdUMP, 5-FdUTP, 5-FUTP) by cellular enzymes. Thus, the series of events, namely irreversible inhibition of thymidylate synthase and formation of (5-FU) RNA and (5-FU) DNA, results in cell death [13,14]. This enzyme is found in several bacteria and fungi but not in mammalian cells [15]. Thus, drug-resistant mammalian cells become sensitive towards 5-FC when transfected with CD.

Although the CD/5-FC system has shown its efficacy in a number of clinical trials, its application in therapy has been limited [16–18]. Despite having various contrasting features, bacterial CD (bCD) still faces setbacks because of its low specificity towards prodrug, 5-FC, thus limiting the overall therapeutic response [19]. It has been shown that yeast cytosine deaminase has better affinity towards 5-FC compared to bCD. However, it is less thermostable than bCD, making bCD a better choice for GDEPT applications. An attempt has been made to create a stable mutant of bCD by random mutagenesis, with marginally improved affinity towards prodrug, 5-FC [20].

During a previous study by our group, computational and genetic engineering approaches were exploited to design bCD mutants with an improved specificity towards prodrug, 5-FC, aiming to overcome the limitation of bCD [21]. Unlike earlier reports on the design of mutants by random mutagenesis [22], we performed site-directed mutagenesis to alter the substrate specificity of bCD, both *in silico* and *in vitro*. Out of these promising mutants, we chose F186W based on kinetic values (Table 1) for study on human lung cancer cells (A549). The aim of the present study was to investigate the activity of the mutant F186W/5-FC on lung cancer cells and its potency in comparison with wild-type bCD (wtCD). The results obtained demonstrate that this mutant significantly enhanced the therapeutic efficacy of CD/5-FC mediated suicide gene therapy on A549 cells and has the potential to emerge as a substitute for wtCD.

Table 1. Relative specificity of wtCD and F186W enzymes [21]

Enzyme	Specificity with respect to 5-FC	Relative to wtCD
wtCD	0.094	1.0
F186W	0.556	5.95

Materials and methods

Cell line and culture conditions

Adenocarcinomic human alveolar basal epithelial cell line (A549) was procured from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/ml; all from Sigma-Aldrich, St Louis, MO, USA), at 37 °C in humidified air containing 5% CO_2. Treatments with drug 5-FC were performed (usually for 72 h) at various concentrations.

Construction of mammalian expression vector

The wtCD and F186W mutants were subcloned from pGEX-4 T2 (Amersham Bioscience, Piscataway, NJ, USA) containing wtCD and F186W into the mammalian expression vector, pVITRO2-hygro-GFP/LacZ (Invivogen, Carlsbad, CA, USA). The wtCD and F186W genes were amplified from their corresponding bacterial expression vectors, namely pGEX-wtCD and pGEX-F186W, respectively. The pVITRO2-GFP/wtCD and pVITRO2-GFP/F186W were constructed by replacing the LacZ gene with wtCD and F186W, respectively, by using forward primer 5'-CGTCCATGGGAATGGTGTCGAATAACGC-3' and reverse primer 5'-CCTG CTAGCTTAGCTCCGCTGATACGTTT-3' with *Nco*I and *Nhe*I restriction sites. Furthermore, the constructs were confirmed by restriction enzyme digestion using *Nco*I and *Nhe*I.

Transient transfection and detection of wtCD/F186W expression

A549 cells were transiently transfected using Lipofectamine 3000 reagent (Invitrogen) in accordance with the manufacturer's instructions. The concentrations of DNA and Lipofectamine were kept constant throughout the experiments. After 6 h of transfection, cells were further treated with prodrug. For confirmation of

DOI: 10.1002/jgm

wtCD and F186W expression, the total RNA of the transfected A549 cells (A549-wtCD and A549-F186W) was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Furthermore, 1 μg of RNA was used for the generation of cDNA with the help of the Verso cDNA Kit (Thermo Scientific, Waltham, MA, USA). A semi-quantitative polymerase chain reaction (PCR) was performed with CD primers using cDNA of the wtCD and F186W. β-actin gene expression was used as an internal control. The PCR products were ran on a 1.2% agarose gel and stained with ethidium bromide. As the wtCD and F186W genes were inserted in a GFP expressing vector, the extent of transfection was visualized under a fluorescence microscope after 6 h of transfection.

Assessment of cell viability by the MTT assay

The initial comparative analyses between parental, wtCD and F186W transfected A549 cells were performed by assessing cell viability after 5-FC exposure. A549 cells were seeded in 96-well plates at a density of 7000 cells/well in DMEM medium supplemented with 10% FBS. The cells were allowed to adhere overnight and transfection was performed in serum-free medium. After 6 h of transfection, the serum-free medium was replaced with experimental medium. The cells were exposed to various concentrations of 5-FC (0.05, 0.1, 0.5, 1 and 5 mM) or PBS (control) for 72 h. After the indicated time, the anti-cell proliferative activity of wtCD and F186W was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay (HiMedia, Mumbai, India) [23]. Fractional cell survival at each drug concentration was calculated by measuring absorbance at 550 nm (Bio-Rad, Hercules, CA, USA) and subtracting the background measurement at 650 nm. Cell viability (%) was calculated relative to untreated viable cells. Each experiment was performed in triplicate and at least twice. The proliferation of the control group was set at 100%.

Trypan blue dye exclusion assay

Cells at a density of 1×10^5 were seeded in a six-well plate. After transfected cells were exposed to 5-FC (0.01 and 1 mM) for 72 h, the cells were harvested and mixed with an equal volume of 0.4% trypan blue dye (Invitrogen). Cells were loaded over the counting chamber. Live and healthy cells were unstained or excluded from dye, whereas dead or membrane compromised cells appeared to retain the dye and appeared blue. The viable cells (%) were counted using a Countess-automated cell counter (Invitrogen).

Acridine orange/ethidium bromide dual staining

Morphological identification of live, apoptotic and necrotic cells was performed by dual staining with acridine orange (AO)/ethidium bromide (EtBr) (Sigma-Aldrich). Cells were grown to 70–80% confluency in a 12-well plate. After transfection with wtCD and F186W, cells were treated with 1 mM 5-FC for 74 h. After the indicated times, the media were removed and washed with cold PBS. Fresh PBS containing 2 μg/ml AO and 6 μg/ml EtBr was added to the cells and kept in the dark for 10 min. The cells were then washed thoroughly with fresh PBS and visualized under a fluorescence microscope (Nikon Eclipse Ti-U, Tokyo, Japan; excitation filter of 480/15 nm for AO and 540/25 nm for EtBr, respectively).

Cell cycle analysis

The proportions of cells in different cell cycle phases were determined by flow cytometric analysis of their DNA content. Cells were seeded at a density of 1×10^5 in a six-well plate and left overnight for attachment. The cells maintained in serum-containing media were replaced with serum-free media and left for cell synchronization for 48 h. Subsequently, cells were transfected with wtCD and F186W, and then exposed to 1 mM 5-FC for 48 h. At the end of the treatment period, cells were harvested and fixed in 70% alcohol solution for 15 min in ice. The fixed cells were collected by centrifugation and stained with propidium iodide (PI; Sigma-Aldrich) staining solution (50 μg/ml PI, 0.1 mg/ml RNase A and 0.05% Triton X-100) at 37 °C for 30 min in the dark. Furthermore, the stained cells were analyzed by flow cytometry (FacsCalibur, BD Biosciences, NJ, USA) at 10 000 events each. The various phases of cell cycle were then assessed by CellQuest and ModFit LT software (Verity House Software, Topsham, ME, USA).

Caspase-3 assay for apoptosis detection

For assessing the apoptotic cell population, phycoerythrin (PE) conjugated caspase-3 antibody (BD Biosciences, Clontech, Palo Alto, CA, USA) was used. Cells were grown in a six-well plate at 70–80% confluency. After treatment for 62 h, as described above, cells were harvested with trypsin-ethylenediaminetetraacetic acid. The detached cells were fixed with 0.1% formaldehyde in PBS at 37 °C for 10 min. Fixed cells were centrifuged

at 450 *g* for 6 min at 4 °C and washed with cold PBS. Then, the cells were permealized with 0.5% Tween-20 in PBS for 10 min. Furthermore, the cells were washed with PBS for two times, 20 µl of caspase-3 conjugated with PE antibody (in accordance with the manufacturer's instructions) was added to each sample, before incubation in the dark for 30 min. The samples were then analyzed with flow cytometer (FacsCalibur, BD Biosciences, NJ, USA) at 10 000 events each. The data were analyzed by Cell Quest Pro software (BD Biosciences) in the same instrument.

Statistical analysis

Significant differences between wtCD and F186W during cell viability assay were assessed by two-way analysis of variance using Prism, version 5.01 (GraphPad Software Inc., San Diego, CA, USA). Statistically significant values were: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Results and Discussion

Subcloning and characterization of wtCD and F186W genes

A 1298-bp fragment of each of wtCD and F186W genes, having *Nco*I and *Nhe*I overhangs, was amplified from their respective pGEX-4 T2 bacterial expression vector. The genes were further subcloned into the mammalian expression vector, pVITRO2-hygro-GFP/LacZ, replacing LacZ gene. The clones were confirmed by performing restriction digestion with the enzymes, *Nco*I and *Nhe*I (Figure 1a).

Transient transfection and expression analysis

Mammalian expression vectors containing genes, pVITRO2-GFP/CD and pVITRO2-GFP/F186W, respectively, were

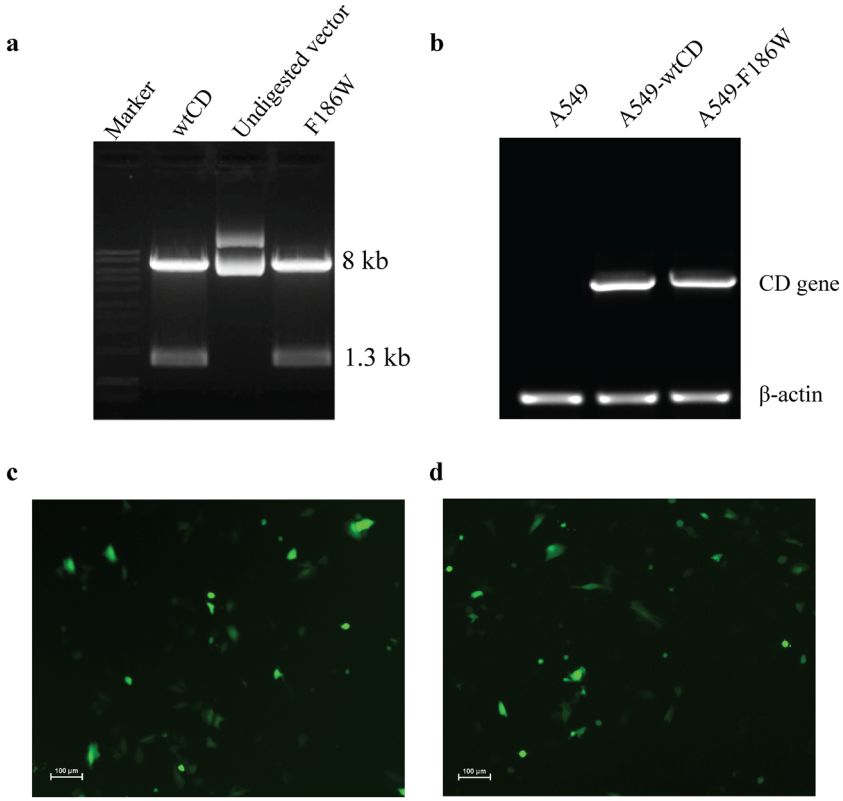

Figure 1. Subcloning, transfection and expression of wtCD and F186W genes. (a) Agarose gel electrophoresis depicted clone confirmation by restriction digestion of wtCD and F186W genes (approximately 1.3 kb) containing vector, using *Nco*I/*Nhe*I restriction enzymes. The undigested vector with a size of approximately 8 kb was taken as the control. (b) Expression of wtCD and F186W genes was evaluated by reverse transcriptase-PCR (RT-PCR) after transfection. β-actin gene was taken as a control. After 6 h of transfection, the expression of GFP was visualized under a fluorescence microscope (scale bar = 200 µm). The GFP expression of wtCD and F186W is shown in (c) and (d), respectively.

DOI: 10.1002/jgm

then transfected in A549 cancer cell lines. Transfection efficiencies were initially assessed by visualizing in fluorescence microscope for GFP expression (Figure 1c and 1d). Furthermore, to examine the expression of wtCD and F186W, the whole RNA from the transfected A549 cells was isolated and cDNA was prepared. Using CD gene-specific primers, expression of wtCD and F186W gene was analyzed by semi-quantitative PCR (Figure 1b). The transfected cell lines showed a distinct band at 1.3 kb, which coincided with the position of CD gene amplified from vector alone (data not shown), whereas, in the untransfected cell line, the band was absent.

Comparative analysis of wtCD and F186W by cell viability assays

To address whether the mutant F186W is more efficient than wtCD, initial analysis was performed by a cell viability assay of wtCD and F186W transfected cell lines, after being exposed to different concentrations of 5-FC. Figure 2 showed the comparative analysis of wtCD and F186W transfected A549 cells, treated with the different concentrations of the prodrug 5-FC. After 72 h of treatment, the data obtained clearly indicated that the F186W was much more effective than wtCD, whereas the parental cells remained unresponsive towards 5-FC exposure.

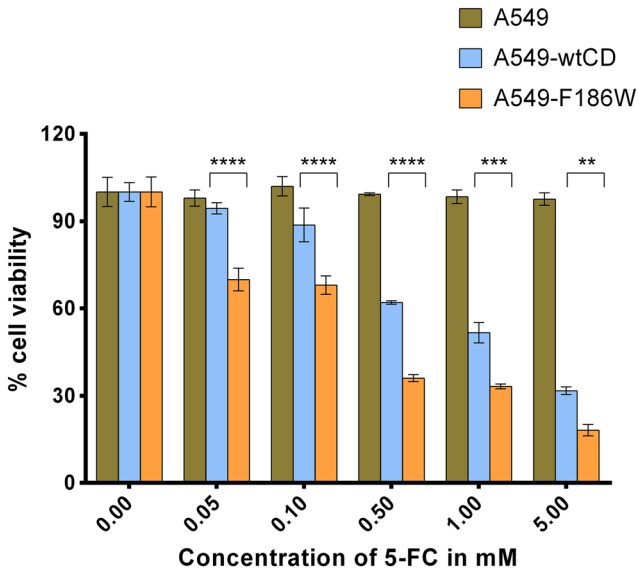

Figure 2. Cell viability assay. A cell viability assay (MTT assay) was performed for initial comparative analysis between wtCD and F186W expressing A549 cells. The data showed a significant advantage of F186W over wtCD. After 72 h of treatment with increasing doses of 5-FC, the resulting data suggested that F186W reached its IC_{50} at a far lower concentration than wtCD. Statistically significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

Therefore, it was observed that the F186W had better 5-FC utilization activity than wtCD. The IC_{50} value of wtCD was found to be at 1 mM 5-FC concentration, whereas, for F186W, it was 0.3 mM 5-FC, which was far lower. Hence, 1 mM (~IC_{50} of wtCD) of 5-FC was chosen for further experimental analysis and comparisons.

Furthermore, the trypan blue dye exclusion assay supported the results obtained from the MTT assay (Figure 3). The trypan blue dye exclusion assay provided a direct identification and calculation of live (unstained; excluded from trypan blue) and membrane compromised or dead cells (blue, stained) in the treated cell population.

Assessment of mode of cell death by dual staining with AO/EtBr

To gain further insight into the manner of cell death, the treated cells were stained with fluorescent DNA intercalating dye namely AO/EtBr and observed under a fluorescence microscope. From Figure 4, it is evident that untreated transfected cells had uniformly stained green nuclei indicating healthy viable cells. When both wtCD and F186W tansfected cells were treated with an IC_{50} dose of wtCD, green, orange and red nuclei were observed, implying the presence of live, early and late apoptotic cells. However, by analyzing the cell population microscopically, it could be inferred that the F186W-containing cell lines had much more apoptotic cells than wtCD harbouring cells. Thus, the AO/EB staining results showed that the possible induction of apoptosis was greater in F186W cell lines.

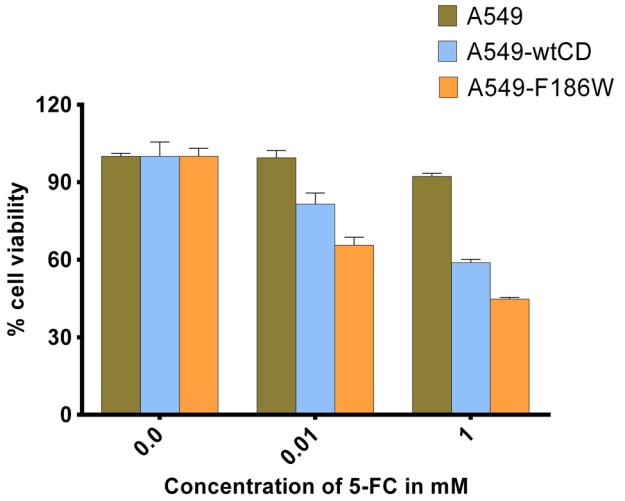

Figure 3. In further support of the experimental data obtained from the MTT assay, a trypan blue dye exclusion assay was performed.

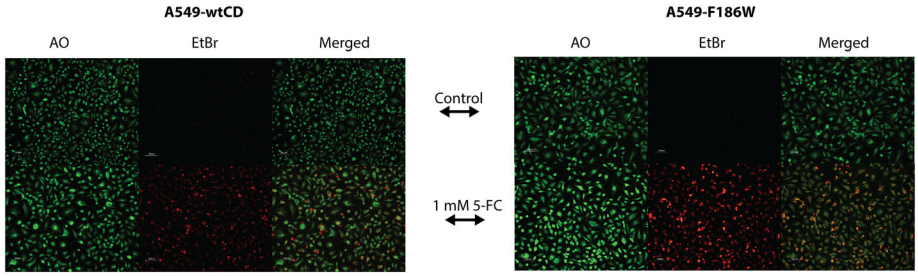

Figure 4. Assessment of mode of cell death. Representative image of AO/EtBR dual staining: the transfected cells were treated with 1 mM 5-FC for 72 h. At the end of the treatment, cells were stained with AO/EtBr. The upper images correspond to transfected but untreated stained cells and the lower image represents treated cells (1 mM 5-FC). The AO and EtBr stained cell images were merged to distinguish between live (green), early apoptotic (orange) and dead (red) cells F186W transfected cells, when treated with 1 mM 5-FC, showed a large number of apoptotic nuclei compared to wtCD transfected cells. Scale bar = 200 μm.

5-FC induces G1 arrest in CD transfected cells

5-FU induced G1 arrest in cells was reported previously [24]. CD catalysed 5-FC conversion to 5-FU may also be attributed to the same. The flow cytometric analysis of DNA content of wtCD and F186W transfected cells, after 5-FC treatment, showed G1 arrest. Cells were serum deprived for 48 h, resulting in G1 arrest of cells. Replacement of serum-enriched media led to synchronized

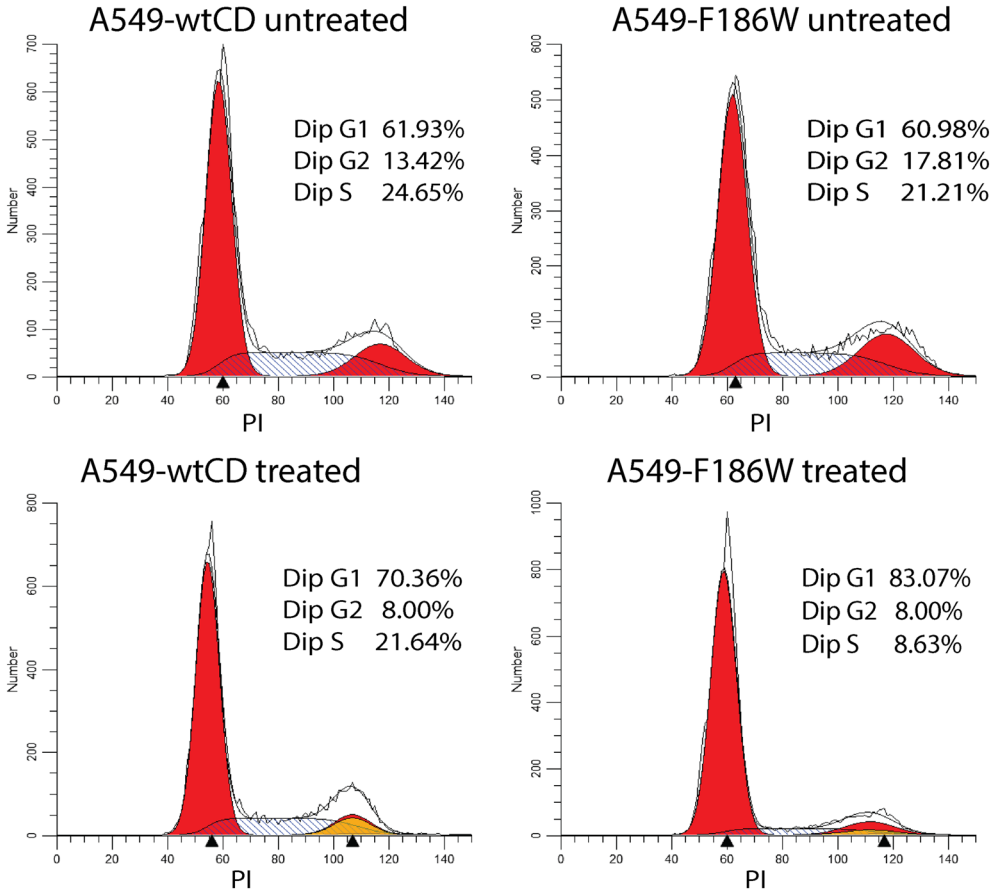

Figure 5. Cell cycle analysis using PI. The synchronized A549 cells were transfected and treated with 1 mM 5-FC for 48 h. Flow cytometric analysis of the DNA content of wtCD and F186W transfected and treated cells revealed that, when treated with 5-FC, the G1 population of wtCD transfected cells was increased to 70.36% compared to the untreated control, which comprised 61.93% of the G1 cell population. Interestingly, F186W showed 83.07% of cells arrested in the G1 phase of cell cycle, which was 13.66% higher compared to wtCD.

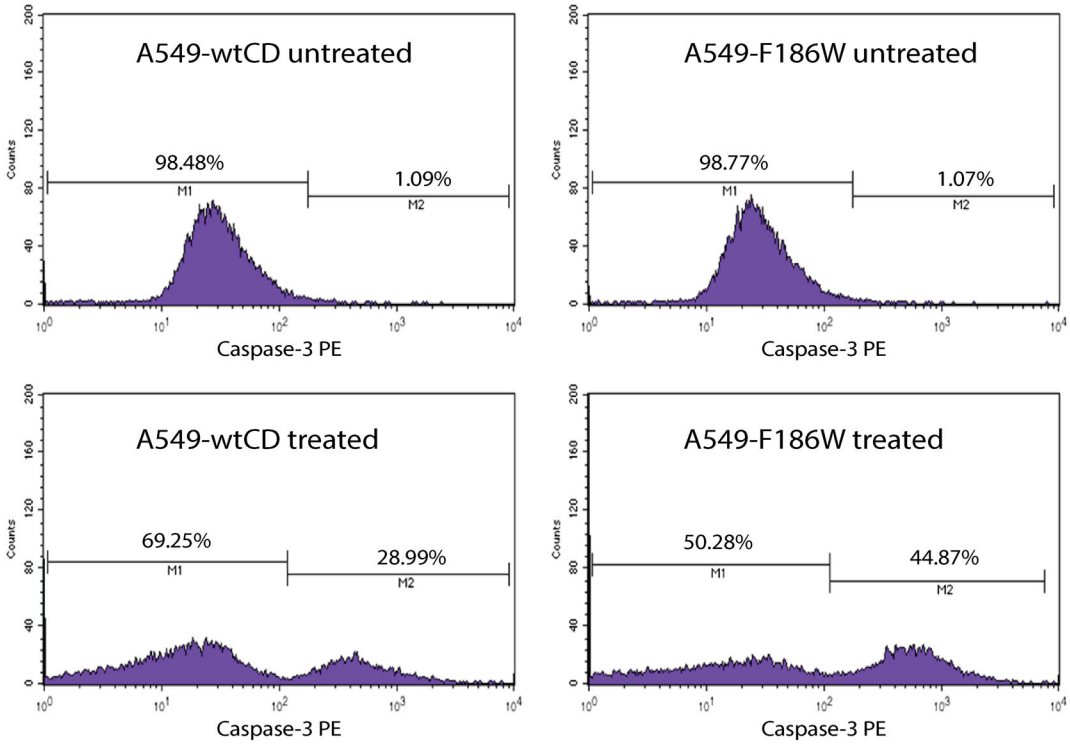

Figure 6. Caspase 3 assay for the progression and execution of apoptosis. wtCD and F186W transfected A549 cells were treated with 1 mM 5-FC for 72 h. The flow cytometric analysis of active caspase 3 conjugated with PE revealed that the treated F186W-A549 cells comprised a 1.5-fold more apoptotic cell population compared to wtCD cells.

progression of cells into the S-phase of the cell cycle. Exposure of parental cells towards 5-FC resulted in no significant cell cycle arrest. wtCD and F186W transfected cells, when treated with 5-FC, remarkably increased the G1 population of cells (Figure 5). However, the percentage of cells in F186W transfected cells was much more pronounced than the wtCD one. Thus, it could be concluded that the conversion of 5-FC to 5-FU by F186W mutant was more compelling than wtCD, supporting previously reported *in vitro* data [21]. The apoptotic cell population was excluded when analysing treated cells using ModFit LT software. Furthermore, for the analysis of apoptotic cells, the caspase 3 assay was performed.

Caspase 3 activity was more pronounced in F186W expressing cells than wtCD expressing cells

Caspase 3 cleavage is a crucial evidence for the progression and execution of apoptosis [25]. F186W transfected cells, when exposed to 5-FC, showed 44.87% caspase 3 activity compared to the parental cell line. On the other hand, the wtCD transfected cells depicted only 28.99% of caspase 3 activity (Figure 6) under similar conditions. Thus, our experimental data provided a clear insight into the mode of cell death and the comparative analysis of the activity of wtCD and F186W. Caspase 3 activity in F186W-A549 cells was increased by approximately 1.5-fold compared to wtCD-A549 cells. It was previously established that 5-FU, generated from 5-FC by CD, substantially mediates bystander effects irrespective of gap junction [26]. As F186W-A549 achieved an IC50 value with a much lower concentration of 5-FU, it may be inferred that the bystander effect is much more prominent in mutant than wtCD-A549, at the same time as minimizing the side effects of a high concentration of 5-FC [27].

Conclusions

In summary, a simple transfection based experiment established that the redesigned F186W mutant has superior therapeutic potency compared to the wtCD. Our experimental results suggest that the F186W is significantly more efficient in utilizing prodrug 5-FC and converting it into toxic metabolite 5-FU when expressed in a mammalian cancer cell line. Because a higher 5-FC drug dose leads to systemic side effects as a result of conversion of 5-FC into 5-FU by intestinal microflora [27], our study provides an alternate mutant, which has high affinity towards 5-FC, thus requiring a much lower dose

DOI: 10.1002/jgm

of 5-FC to achieve IC_{50}. Our experimental analysis on computationally designed mutant F186W has established the mutant enzyme as a superior candidate in cancer therapeutics. Hence, F186W mutant will be more suitable than wtCD for further gene therapy applications.

Acknowledgements

We acknowledge the Department of Biotechnology and the Department of Electronics and Information Technology [No. 5 (9)/2012-NANO (Vol. II)], Government of India for their financial support. We also acknowledge the aid of the Central Instruments Facility (CIF) and the Centre for Nanotechnology, IIT Guwahati. All authors decalre that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in the present study. No human and/or animals are involved in the present study. The authors have provided consent for submission and publication.

References

1. Springer CJ, Niculescu-Duvaz I. Prodrug-activating systems in suicide gene therapy. *J Clin Invest* 2000; **105**: 1161–1167.
2. Matuskova M, Hlubinova K, Pastorakova A, *et al.* HSV-tk expressing mesenchymal stem cells exert bystander effect on human glioblastoma cells. *Cancer Lett* 2010; **290**: 58–67.
3. Li Bi W, Parysek LM, Warnick R, Stambrook PJ. In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. *Hum Gene Ther* 1993; **4**: 725–731.
4. Asklund T, Appelskog IB, Ammerpohl O, *et al.* Gap junction-mediated bystander effect in primary cultures of human malignant gliomas with recombinant expression of the HSVtk gene. *Exp Cell Res* 2003; **284**: 183–193.
5. 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 U S A* 1996; **93**: 1831–1835.
6. 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.
7. Motohiro M, Toshiya S, Shusuke M, Joseph CG, Masami B. In vitro thymidine kinase/ganciclovir-based suicide gene therapy using replication defective herpes simplex virus-1 against leukemic B-cell malignancies (MCL, HCL, B-CLL). *Leuk Res* 2003; **27**: 695699.
8. Soubrane C, Mouawad R, Rixe O, *et al.* Direct gene transfer of a plasmid carrying the herpes simplex virus-thymidine kinase gene (HSV-TK) in transplanted murine melanoma: in vivo study. *Eur J Cancer* 1996; **32A**: 691–695.
9. Lv S-QQ, Zhang K-BB, Zhang EE, *et al.* Antitumour efficiency of the cytosine deaminase/5-fluorocytosine suicide gene therapy system on malignant gliomas: an in vivo study. *Med Sci Monit* 2009; **15**: 20.
10. 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 U S A* 1992; **89**: 33–37.
11. McMasters RA, Saylors RL, Jones KE, *et al.* Lack of bystander killing in herpes simplex virus thymidine kinase-transduced colon cell lines due to deficient connexin43 gap junction formation. *Hum Gene Ther* 1998; **9**: 2253–2261.
12. Kuriyama S, Masui K, Sakamoto T, *et al.* Bacterial cytosine deaminase suicide gene transduction renders hepatocellular carcinoma sensitive to the prodrug 5-fluorocytosine. *Int Hepatol Commun* 1995; **4**: 72–79.
13. Springer CJ, Niculescu-Duvaz I. Gene-directed enzyme prodrug therapy (GDEPT): choice of prodrugs. *Adv Drug Delivery Rev* 1996; **22**: 351–364.
14. Moolten FL. Drug sensitivity ('suicide') genes for selective cancer chemotherapy. *Cancer Gene Ther* 1994; **1**: 279–287.
15. Ireton GC, McDermott G, Black ME, Stoddard BL. The structure of *Escherichia coli* cytosine deaminase. *J Mol Biol* 2002; **315**: 687–697.
16. Crystal RG, Hirschowitz E, Lieberman M, *et al*. Phase I study of direct administration of a replication deficient adenovirus vector containing the *E. coli* cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum Gene Ther* 1997; **8**: 985–1001.
17. Nemunaitis J, Cunningham C, Senzer N, *et al.* Pilot trial of genetically modified, attenuated *Salmonella* expressing the *E. coli* cytosine deaminase gene in refractory cancer patients. *Cancer Gene Ther* 2003; **10**: 737–744.
18. Pandha HS, Martin L-A, Rigg A, *et al.* Genetic prodrug activation therapy for breast cancer: a phase I clinical trial of erbB-2-directed suicide gene expression. *J Clin Oncol* 1999; **17**: 2180.
19. Kievit E, Bershad E, Ng E, *et al.* Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. *Cancer Res* 1999; **59**: 1417–1421.
20. Mahan SD, Ireton GC, Knoeber C, Stoddard BL, Black ME. Random mutagenesis and selection of *Escherichia coli* cytosine deaminase for cancer gene therapy. *PEDS* 2004; **17**: 625–633.
21. Kohila V, Jaiswal A, Ghosh SS. Rationally designed *Escherichia coli* cytosine deaminase mutants with improved specificity towards the prodrug 5-fluorocytosine for potential gene therapy applications. *MedChemComm* 2012; **3**: 1316–1322.
22. Fuchita M, Ardiani A, Zhao L, *et al.* Bacterial cytosine deaminase mutants created by molecular engineering demonstrate improved 5FC-mediated cell killing in vitro and in vivo. *Cancer Res* 2009; **69**: 4791–4799.
23. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936–942.
24. Sun X-X, Dai M-S, Lu H. 5-Fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. *J Biol Chem* 2007; **282**: 8052–8059.
25. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999; **6**: 99–104.
26. Kuriyama S, Masui K, Sakamoto T, *et al.* Bystander effect caused by cytosine deaminase gene and 5-fluorocytosine in vitro is substantially mediated by generated 5-fluorouracil. *Anticancer Res* 1998; **18**: 3399–3406.
27. Harris BE, Manning BW, Federle T, Diasio RB. Conversion of 5-fluorocytosine to 5-fluorouracil by human intestinal microflora. *Antimicrob Agents Chemother* 1986; **29**: 44–48.