Connexin-43 Enhances the Redesigned Cytosine Deaminase Activity for Suicide Gene Therapy in Human Breast Cancer Cells

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ABSTRACT

BACKGROUND: Escherichia coli cytosine deaminase (CD) converts 5-fluorocytosine (5-FC), a prodrug, into 5-fluorouracil (5-FU), a chemotherapeutic drug. However, the poor binding affinity of CD towards 5-FC as compared to the natural substrate cytosine, limits its application towards a successful suicide gene therapy. Although F186W mutant was developed to enhance the effect of wild-type CD, still scope for its improvement remains to further minimize the dose-dependent cytotoxicity of the drugs. Hence, in this study, we employ the anti-tumour attribute of the gap junction forming protein connexin-43 (Cx43) in conjunction with CD or F186W mutant.

METHODS: Lipofectamine was used to co-transfect CD/F186W-pvITRO2 and Cx43-pEGFP-N1 plasmids construct into MCF-7 cells. Comparative analysis of cell viability was observed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) and trypan blue-based assays. To further confirm the mode of cell death was apoptosis, propidium iodide and annexin V/7-aminoactinomycin D (7-AAD)-based apoptosis assays were performed.

RESULTS: Semi-quantitative polymerase chain reaction (PCR) confirmed the expression of both Cx43 and CD/F186W genes after transfection. Furthermore, cell viability assays revealed the enhanced activity of F186W-Cx43 compared with CD-Cx43 and F186W alone. The trend of the reduction in cell viability was also reflected in the flow cytometry–based apoptosis analyses. Overall, F186W-Cx43 combination demonstrated its superiority over the CD-Cx43 and F186W mutant alone.

CONCLUSIONS: The enhanced cytotoxic activity of F186W mutant was further amplified by gap junction protein Cx43.

KEYWORDS: connexin-43, cytosine deaminase, suicide gene therapy, gap junction protein, gene therapy

Introduction

Gene therapy has shown an immense potential in the field of the most advanced and novel approaches to treat various cancers with less adverse effects. One particularly appealing approach is to transfer and expression of a suicide gene in the tumour environment (suicide gene therapy; SGT). Although SGT participated in various clinical trials, the scope of its improvement is continuously evolving.1 Gene-directed enzyme prodrug therapy (GDEPT) or molecular chemotherapy is based on the principles of SGT, which incorporates the intratumoural delivery of suicide genes. Once inside the cell, suicide gene encodes a nonmammalian enzyme which activates nontoxic prodrug into a cytotoxic drug, which further triggers the apoptosis in the cells.2 The GDEPT combination of Escherichia coli cytosine deaminase (CD; EC 3.5.4.1) with antifungal drug 5-fluorocytosine (CD/5-FC) may be used to circumvent the pharmacokinetic limitations of systemic 5-fluorouracil (5-FU).3 The CD/5-FC system showed various advantages over other GDEPT systems. In particular, 5-FU can act as both, a cytotoxic drug and as a radiosensitizer.4 This makes the CD/5-FC system a perfect choice for the patients going through radiation treatments. The CD/5-FC system with the brand name Toca 511 (vocimagene amiretrorepvec), a retroviral replicating vector, is in advanced clinical evaluation.5 In another advantage, the CD/5-FC system does not reckon on the presence of gap junction intracellular communication (GJIC) between the cells, as the toxic metabolite 5-FU can readily move across the cell membrane and cause inhibition of the bystander cells not expressing CD gene.6 Although the activity 5-FU generated by CD is independent of GJIC, the anti-tumour property of gap junction protein connexin-43 (Cx43) still helps in increasing the efficacy of the CD/5-FC system. Connexin-43 exerts a dual role in suppressing tumours, by allowing bystander killing of the neighbouring cells through gap junction and by regulating the pro-apoptotic genes in the cancer cells.7

Despite having several contrasting features of the CD/5-FC system, its use in the clinic has been limited due to low specificity and activity of bacterial CD or wild-type CD towards 5-FC. To circumvent the said limitations, a CD mutant was designed...
in our laboratory. The CD mutant, named F186W, have proven its enhanced specificity and activity in the cell line–based system. The aim of this study was to further enhance the F186W mutant activity by co-transfecting it with the Cx43 gene in MCF-7 cells. The results obtained demonstrated that the expression of the Cx43 protein in the MCF-7 cells led to the increase in the dose-dependent cytotoxicity of the CD and F186W mutant activity. However, F186W mutant showed more pronounced enhancement in the therapeutic efficacy.

Materials and Methods

Cell line and culture conditions

Human breast adenocarcinoma (MCF-7) cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. High glucose Dulbecco’s modified Eagle’s medium (DMEM) was used to culture the cells, which is supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/mL). The cells were grown in humidified air containing 5% CO2 at 37°C.

Construction of plasmid

pVTR2-hygro-GFP/LacZ (Invivogen, USA) was used to construct the mammalian expression vector containing the CD and F186W mutant genes, as described by Raza et al. The previously designed Cx43-pEGFP-N1 mammalian expression was used by Raza et al. Sham-transfected cells were analysed before for any adverse cell cytotoxicity.

Co-transfection and expression analysis of Cx43, CD, and F186W gene expression

Cells were stably transfected as per the maker’s protocol using Lipofectamine 3000 reagent (Invitrogen, Karlsruhe, Germany). Control and transfected cells were seeded at a density of 7000 cells/well in a 96-well plate. After 24 h of incubation, the cells were transfected in reduced serum media. Selection of the stably transfected cell was performed using 300 µg/mL G418 (Sigma–Aldrich, Germany) and 100 µg/mL hygromycin (HiMedia, India) for Cx43 and CD or F186W, respectively. A semi-quantitative PCR was performed to investigate the expression of Cx43, CD, and F186W genes by isolating the total RNA of the transfected cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich). Complementary DNA (cDNA) was generated with the help of Verso cDNA Kit (Thermo Scientific, MA, USA) taking 1 µg of RNA. Polymerase chain reaction (PCR) was performed using Cx43 and CD primers, taking cDNA pool of the transfected cell line as a template. Polymerase chain reaction conditions used were as follows: initial denaturation at 94°C for 2 min was followed by 35 PCR cycle of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide. The β-actin gene expression was taken as endogenous control.

GJIC functional assay

The functional GJIC was assessed by dye transfer assay using PKH26 and calcein-acetoxyethyl (AM) ester dyes. MCF-7 and Cx43–MCF-7 were grown in a six-well plate to their confluence. Connexin-43–MCF-7 cells were labelled with 5 µM calcein-AM (30 min at 37°C) and 2 µM PKH26 (10 min at 25°C) dyes. Dual-stained cells were trypsinised and plated on top of the cells grown in a 12-well plate at a ratio of 1:50 (labelled:unlabelled). After 6 h of incubation, the cells were visualized under the fluorescence microscope (Nikon Eclipse Ti-U, Tokyo, Japan).

Cell viability assay

The CD or F186W mutants were transfected in Cx43–transfected MCF-7 cells. The cells were then treated with 5-FC, and cell viability was assessed as follows. A total of 7 × 10^4 cells/well of 96-well plate were used to assess the dose-dependent cell viability of the transfected cells. After assessment of successful transfection, cells were treated with the varying concentrations of 5-FC or phosphate-buffered saline (PBS; control) for 72 h. After the indicated time, the decrease in cell viability of the treated cells was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay (HiMedia). Percentage cell viability was investigated by taking absorbance of the formazan compound formed at 570 nm (Infinite 200 PRO, Tecan, Crailsheim, Germany) and normalizing the background measurement at 650 nm at each drug concentration. Data were normalized by taking cell viability of untreated cells as 100%.

Trypan blue dye exclusion assay

Trypan blue dye was used to quantify the live and dead cells after treatment with 1 mm of 5-FC. Cells were grown at a density of 1 × 10^5 cells/well of a six-well plate. After treatment with 5-FC for 72 h, cells were trypsinised and stained with 0.4% trypan blue dye (Invitrogen). The trypan blue–stained cells were put into the Countess cell-counting chamber slides. Live and healthy cells remained unstained or excluded from the trypan blue, while membrane compromised or dead cells appeared blue due to trypan blue retention. The percentage of live and dead cells was calculated using Countess–automated cell counter (Invitrogen).

Fluorimetric analyses of apoptotic cells using propidium iodide and phycoerythrin Annexin V/7-aminoactinomycin D

The dead cell population was analysed using the protocol described earlier. MCF-7 cells were treated with 5-FC for 72 h. After the completion of the treatment duration, the cells were collected, fixed in 70% ethanol and processed following the protocol mentioned above. The cells were then analysed using CytoFLEX flow cytometer (Beckman Coulter, Miami,
FL) using 488 nm laser line for excitation. Red fluorescence of propidium iodide (PI) was measured in PE-A channel.

The different apoptotic cell populations were quantified using PE Annexin V and 7-aminoactinomycin D (7-AAD), following treatment with 5-FC. In brief, cells were grown and treated with 1 mM of 5-FC for 72 h. After the end of the indicated time, the cells were collected by trypsinization and stained with PE Annexin V and 7-AAD, following the manufacturer’s protocol (BD Biosciences, Heidelberg, Germany). Furthermore, the extent of apoptosis in the cells was analysed using flow cytometer (CytoFLEX, Beckman Coulter).

**Cell cycle analysis**

Assessment of various cell cycle phases was determined by analysing the DNA content of the cells using flow cytometer. For that, cells were grown at a density of 1 × 10⁵ cells/well in a six-well plate. After 24 h of incubation, the cells were synchronized in G1 phase by serum starvation for 24 h. Subsequently, cells were supplemented with serum medium and treated with 1 mM of 5-FC for 48 h. After the completion of the treated duration, cells were detached, fixed using 70% chilled alcohol and labelled with PI (Sigma-Aldrich) staining solution (50 μg/mL PI, 0.1 mg/mL RNase A, and 0.05% Triton X-100) in the dark at 37°C for 20 min. The PI-labelled cells were acquired using CytoFLEX (Beckman Coulter) in PE-A channel. ModFit LT software (Verity Software House) was used to analyse the data.

**Statistical analysis**

Data points were expressed as the mean ± SD (SD = standard deviation) and experiments were performed at least thrice in triplicates. The two-way analysis of variance (ANOVA) was used for pairwise comparisons to assess the statistical significance of differences. Statistically significant values (P-value) for ANOVA corresponds to *P < .05, **P < .01, ***P < .001, and ****P < .0001.

**Results**

**Expression of Cx43 in MCF-7 cells and co-transfection of Cx43 with CD/F186W gene**

The Cx43-pEFGP-N1 plasmid was transfected using lipofectamine into MCF-7 cells. Semi-quantitative PCR using Cx43-specific primers amplified a gene of 1.2 kb from the cDNA pool of Cx43-MCF-7 cells lysate, which corresponds to the coding region of Cx43 gene (Figure 1A). The above PCR-based amplification of Cx43 gene provided an evidence for the successful transfection and expression of Cx43 gene, which led to the confirmation of the expression of Cx43 mRNA. Furthermore, CD or F186W gene containing pVITRO2 mammalian expression vector was then stably transfected into Cx43-MCF-7 cells.

After the stable cell line was generated having both Cx43 and CD/F186W gene, they were screened for the mRNA expression. A discrete band of 1.3 kb in the transfected cell lines (Figure 1B) concurred with amplified CD gene from the pVITRO2-hygro-GFP-CD vector. The untransfected cell line showed no amplification of CD gene.

The functionality of the GJIC after Cx43 transfection was assessed by dye transfer assay (Figure 1C). MCF-7 and Cx43-MCF-7 cells were labelled with two different fluorescent dyes, PKH26, and calcein-AM. Dual-labelled MCF-7 cells or Cx43-MCF-7 cells were co-cultured with unlabelled Cx43-MCF-7 cells by a ratio of 1:50. The extent of dye transfer after 4 h from MCF-7 cells to Cx43-MCF-7 cells was limited to one or no cell without any significant visual dye transfer. However, co-culture of dual-stained Cx43-MCF-7 cells with the plated unlabelled Cx43-MCF-7 cells showed the substantial increase in dye transfer among neighbouring cells, as about 25–30 unlabelled cells showed green fluorescence by sequential transfer of calcein from a single donor cell. PKH26 was retained by both the donor cells without any dye transfer.

**Expression of Cx43 enhanced the suicide gene activity of CD/F186W gene**

To probe into the effect of Cx43 expression in MCF-7 cells on the activity of CD and F186W mutant, MTT-based cell viability assay was performed. Initially, the effect of the prodrug 5-FC was assessed on the MCF-7 and Cx43-MCF-7 cells, and it was found that the 5-FC had no cytotoxic effect on these cells at clinically relevant concentrations (Figure 2A).

MCF-7 and Cx43-MCF-7 cells transfected with CD gene were subjected to 5-FC treatment, a dose-dependent decrease in cell viability was observed (Figure 2B). The CD enzyme formed inside the CD gene-transfected cells converted prodrug 5-FC into cytotoxic drug 5-FU, resulting in the decrease in cell viability. However, the dose-dependent cytotoxicity was more pronounced in the CD-Cx43-MCF-7 cells when compared with CD-MCF-7 cells alone. A similar trend was observed in case of F186W mutant. The F186W-Cx43-MCF-7 mutant showed more sensitivity towards 5-FC treatment as compared to the F186W-MCF-7 cells. However, the F186W-Cx43-MCF-7 cells showed remarkable sensitivity towards 5-FC when compared with CD-Cx43-MCF-7 cells (Figure 2C).

In particular, the toxicity level (measured as IC₅₀) induced by 5-FC was much higher for MCF-7 transfected with CD than Cx43-MCF-7 transfected with CD (Figure 2D). Similarly, a less dose of 5-FC was required to reach IC₅₀ for F186W-Cx43-MCF-7 when compared with F186W-MCF-7. The cell viability–based data showed that the Cx43-transfected MCF-7 cells respond more effectively towards the 5-FC after co-transfecting it with CD/F186W gene compared to MCF-7 cells transfected with CD/F186W. The trend of dose-dependent cell cytotoxicity was further corroborated with trypan blue dye exclusion assay using a constant dose of 1 mM 5-FC (Figure 3). Thus, it can be inferred that Cx43 expression in MCF-7 cells makes them more sensitive towards SGT.
Expression of Cx43 enhanced the induction of apoptosis by 5-FC in co-transfected CD/F186W gene in MCF-7 cells

To establish that 5-FC treated co-transfected MCF-7 cells were undergoing apoptosis, two different sets of flow cytometry-based experiments have been performed. First, the analysis of membrane-compromised cells was done using PI dye and second, the early apoptotic, late apoptotic, and necrotic cells were differentiated using PE Annexin V and 7-AAD assay. Propidium iodide staining is a rapid, reliable, and reproducible method for the separation of membrane-compromised cell population from the live cell population. In our analysis, the extent of cell death mediated by 5-FC on CD-F186W-transfected MCF-7 or Cx43-MCF-7 cells was assessed. After 72 h of treatment, CD-MCF-7 showed 26.95% of the membrane-compromised cell population, and CD-Cx43-MCF-7 cells showed 31.79% of the cell death population. However, F186W-MCF-7 showed 39.05% of the dying cell population, and F186W-Cx43-MCF-7 cells showed 52.02% of the dying cell population (Figure 4).

To further validate the data generated by the PI apoptotic assay, PE Annexin V and 7-AAD flow cytometric assay (Figure 5) was performed. After the end of the treatment duration, the cells were stained with PE Annexin V and 7-AAD. Flow cytometry data corroborated with the above finding that Cx43 enhanced the cytotoxic effect of CD and F186W. About 25.58% and 62.05% of the apoptotic cell population (early and late apoptotic combined) were found after treatment with 5-FC on CD-MCF-7 and CD-Cx43-MCF-7 cells, respectively. When F186W mutant was used in place of CD, the apoptotic populations of treated F186W-MCF-7 and F186W-Cx43-MCF-7 cells were enhanced to 35.7% and 76.07%, respectively.

Expression of Cx43 induced G1 arrest after treatment with 5-FC in co-transfected CD/F186W gene in MCF-7 cells

Perturbation in the cell cycle of the treated cell population was investigated by analysing the DNA content of the cells using PI. Figure 6 showed the flow cytometry data analysed in ModFit LT software, showed a substantial cell population present in the G1 phase of the cell cycle after treatment with 5-FC, as evident earlier.10 The CD-Cx43-MCF-7 cells, when treated with 5-FC, showed 79.53% of the G1
population, while for CD-MCF-7, the population was just 56.37%. Connexin-43 helped in the G1 arrest of the MCF-7 cell population mediated by 5-FU, which was formed from 5-FC after conversion with the help of CD enzyme. In a similar manner, F186W when co-transfected with Cx43 in MCF-7 cells caused a substantial increase in the G1 arrest population, that is, 90.11% as compared to 74.31% for F186W-MCF-7. Overall, F186W mutant was much more effective in arresting cell population in G1 phase relative to CD, and its effectiveness enhanced drastically when it was co-transfected with Cx43 gene.

Discussion
Despite the prominent use of 5-FU in progressive colorectal cancer, the response rate is merely 10%–20%. Multiple strategies, involving the amalgamation of 5-FU with other chemotherapeutic drugs, anti-tumour proteins, radiation therapy, or with cytokines, are implored to enhance the sensitivity of the cancer cells towards 5-FU.12–14 The CD/5-FC gene transfer approach is based on the fact that 5-FU is generated from the prodrug 5-FC by the enzyme CD. Thus, incorporating the gene-encoding CD into the cancer cells could render them more sensitive towards 5-FU treatment. In spite of having the contrasting feature of targeted cell killing, bacterial CD suffered from the major drawback. It is less efficient in converting 5-FC into the cytotoxic drug 5-FU, around 20-fold lesser, when compared with that of its natural substrate cytosine.15 In some cases, to reach a desirable response, the 5-FC doses have been increased, which led to the several side-effects.16 To circumvent the dose-dependent side-effects of the bacterial CD, a CD mutant F186W was designed in our laboratory.17 The F186W mutant showed its superior therapeutic potency in A549 cells by demonstrating its high affinity towards 5-FU.8 It was more efficient in converting the prodrug 5-FC into the cytotoxic drug 5-FU, thus, minimizing the dose-dependent side-effects of 5-FC. In this report, we further
examined the enhancement in the SGT mediated by F186W/5-FC system by combining it with the gap junction forming protein, Cx43.

In our study, we chose Cx43 gene as a co-therapy system in conjunction with CD-based SGT. A plethora of studies has indeed unveiled that loss of GJIC has been predominant in the most of the cancer types, and it was due to lack of connexin expression.\(^{18}\) MCF-7, a human breast adenocarcinoma cell line, used in our study is devoid of Cx43 expression and functional GJIC.\(^{19}\) The forced expression of Cx43 in MCF-7 cells made it susceptible to various chemotherapeutic treatment.\(^{20}\) It was evident that Cx43 acts as an anti-tumour protein by means of gap junction dependent as well as independent pathways.\(^{7,20–23}\) Taken together, we presume that expression of Cx43 gene with the CD/F186W gene could make the MCF-7 cells more susceptible for the SGT.

To confirm the hypothesis, the CD/F186W gene and Cx43 gene were co-transfected in the MCF-7 cells and then classified based on the different combinations of gene expression, these were, MCF-7, CD-MCF-7, Cx43-MCF-7, CD-Cx43-MCF-7, F186W-MCF-7, and F186W-Cx43-MCF-7 cells. The expression of Cx43 and CD/F186W gene were confirmed by using semi-quantitative PCR as shown in Figure 1. The initial analysis of cell cytotoxicity by each of the MCF-7 cell type, cell viability assay was performed. Cell viability data and the resulting IC\(_{50}\) calculation revealed that the expression of CD/F186W gene with the Cx43 gene indeed enhanced its efficacy. However, the enhancement in the cell cytotoxicity was more
prominent in case of F186W-Cx43 combination than CD-Cx43 combination. The IC$_{50}$ value of F186W-Cx43 was 0.18 mM, while for CD-Cx43 it was 2.03 mM. Similar trend were observed in the PI and Annexin V/7-AAD-based apoptosis analysis.

Overall, a simple co-transfection-based system has shown that the potentiality of the F186W mutant was increased when it was transfected with the Cx43 gene. Connexin-43 gene expression led to the enhancement in the efficacy of both CD and F186W gene; however, it was more pronounced in case of mutant as the later was more effective in conversion of 5-FC to 5-FU. Previous study by our group$^3$ delineated that the overexpression of Cx43 in MCF-7 cells could enhance the sensitivity of MCF-7 cells towards chemotherapeutic drugs underlying mechanism associated with it. Experimental data based on the comparative analyses suggested that the co-transfection of F186W in Cx43-MCF-7 cells make it more susceptible to apoptosis after 5-FC treatment than F186W-MCF-7 cells. The additive effect of comparatively more efficient F186W mutant and the anti-tumour protein Cx43, provided an excellent model to enhance the SGT. As higher dose of non-toxic drug 5-FC leads to the systemic side-effect due to conversion of 5-FC into 5-FU mediated by intestinal microflora,$^{10}$ this study renders an efficient co-therapy system, which has high affinity towards 5-FC, making MCF-7 cells more susceptible towards chemotherapeutic drugs and thus, minimal dose of 5-FC is required to attain IC$_{50}$ (Figure 2D).

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**Authors Contributions**

A.R. designed and performed the experiments, interpreted the data, wrote the manuscript, and prepared the figures. S.S.G. supervised the whole project and provided critical revision for content of the manuscript.

**Data Sharing**

The data sets used and/or analysed during this study are available from the corresponding author on reasonable request.

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