

Research Article

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

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Abstract

Background: The redesigned *E. coli* Cytosine Deaminase (CD) mutant F186W provided an excellent alternative to wild-type CD in terms of efficiency and activity. Although the redesigned F186W mutant demonstrated its superiority over the CD, there is still scope for its improvement remains to further minimize the dose-dependent cytotoxicity of the drugs. Hence, in the present study, we employ the anti-tumour attribute of the gap junction forming protein 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. A dose-dependent decrease in cell viability was observed using MTT and trypan blue based assays. To further confirm the mode of cell death was apoptosis, propidium iodide and annexin V/7-AAD based apoptosis assays were performed.

Results: Semi-quantitative PCR confirmed the expression of both Cx43 and CD/F186W genes after transfection. Further cell viability assays showed the enhanced activity of F186W-Cx43 compared to CD-Cx43 and F186W alone. The trend of the reduction of 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.

Conclusion: The enhanced cytotoxic activity of F186W mutant was further amplified using anti-tumor 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 tumor environment (Suicide Gene Therapy; SGT). Although SGT participated in various clinical trials, the scope of its improvement is continuously evolving [1]. Gene-Directed Enzyme Pro Drug Therapy (GDEPT) or molecular chemotherapy is based on the principles of suicide gene therapy, which incorporates the intra tumoral delivery of suicide genes. Once inside the cell, suicide gene encodes a non mammalian enzyme which activates non-toxic pro drug 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 radio sensitizer [4]. This makes the CD/5-FC system a perfect choice for the patients going through radiation treatments. 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 [5]. Although the activity 5-FU generated by CD is independent of GJIC, the anti-tumor property of gap junction protein Connexin-43 (Cx-43) still helps in increasing the efficacy of the CD/5-FC system. Cx-43 exerts a dual role in suppressing tumors, by allowing bystander killing of the neighboring cells through gap junction and by regulating the pro-apoptotic genes in the cancer cells [6]. 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 [7]. The aim of the present study was to further enhance the F186W mutant activity

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by co transfection 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 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% Fetal 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

pVITRO2-hygro-GFP/LacZ (Invivogen, USA) was used to construct the mammalian expression vector containing the CD and F186W mutant genes, as described in [7]. The previously designed Cx43-pEGFP-N1 mammalian expression was used in this study [6].

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). Control and transfected cells were seeded at a density of 7,000 cells/well in a 96 well plate. After 24 hr 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) and 100 µg/ml hygromycin (HiMedia) 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). cDNA was generated with the help of Verso cDNA Kit (Thermo Scientific) taking 1 µg of RNA. PCR was performed using Cx43 and CD primers taking cDNA pool of the transfected cell line as a template. The β-actin gene expression with ethidium bromide.

Cell viability assay

The CD or F186W mutant was transfected in Cx43 transfected MCF-7 cells. The cells were then treated with 5-FC and cell viability was assessed as follows. 7 × 10³ cells per 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 PBS (control) for 72 hr. 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 (Tecan Infinite 200 PRO) 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 quantitate the live and dead cells after treatment with 1 mM of 5-FC. Cells were grown at a density of 1 × 10⁵ cells/well of a 6-well plate. After treatment with 5-FC for 72 hr, 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 were remain unstained or excluded from the trypan blue while membrane compromised or dead cells appeared blue due to trypan blue

retention. The percentages of live and dead cells were counted using Countess-automated cell counter (Invitrogen).

Fluorimetric analyses of apoptotic cells using PI and PE annexin V/7-AAD

The apoptotic population of the cells was analyzed using the protocol described earlier [8]. MCF-7 cells were treated with 5-FC for 72 hr. 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 analyzed by using CytoFLEX flow cytometer (Beckman Coulter) using 488 nm laser line for excitation. Red fluorescence of PI was measured in PE-A channel. The different apoptotic cell populations were quantified using PE (Phycoerythrin) Annexin V and 7-AAD (7-Aminoactinomycin D), following treatment with 5-FC. In brief, cells were grown and treated with 1mM of 5-FC for 72 hr. 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). Further, the extent of apoptosis in the cells was analyzed using flow cytometer (CytoFLEX, Beckman Coulter).

Cell cycle analysis

Assessment of various cell cycle phases was determined by analyzing the DNA content of the cells using flow cytometer. For that, cells were grown at a density of 1 × 10⁵ cells/well in a 6-well plate. After 24 hr of incubation, the cells were synchronized in G1 phase by serum starvation for 24 hr. Subsequently, cells were supplemented with serum medium and treated with 1 mM of 5-FC for 48 hr. After the completion of the treated duration, cells were detached, fixed using 70% chilled alcohol and labelled with propidium iodide (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 was acquired using CytoFLEX (Beckman Coulter) in PE-A channel. ModFit LT software (Verity Software House) was used to analyze the data.

Statistical analysis

Data points were expressed as the mean ± SD (Standard Deviation) and experiments were performed at least thrice in triplicates. The two-way Analysis of Variance (ANOVA) was used for pair wise comparisons to assess the statistical significance of differences. Statistically significant values (p-value) for ANOVA corresponds to *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

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

The Cx43-pEGFP-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. Further, CD or F186W gene containing pVITRO2 mammalian expression vector was then stably transfected into Cx43-MCF7 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 concurred with amplified CD gene from the pVITRO2-hygro-GFP-CD vector (Figure 1b). The un-transfected cell line showed no amplification of CD gene.

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 pro drug 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 pro drug 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 try pan 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 makesthem more sensitive towards suicide gene therapy.

Expression of Cx43 enhanced the induction of apoptosis by 5-FC in co-transfected CD/F186W gene in MCF-7 cells

In order 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 apoptotic nuclei 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.

PI staining is a rapid, reliable and reproducible method for the separation of an apoptotic cell population from the live cell population cell population from the live cell population. In our analysis, the extents of apoptosis mediated by 5-FC on CD/F186W transfected MCF7 or Cx43-MCF-7 cells were assessed. After 72 hr of treatment, CD-MCF-7 showed 26.95% of the apoptotic cell population and CD-

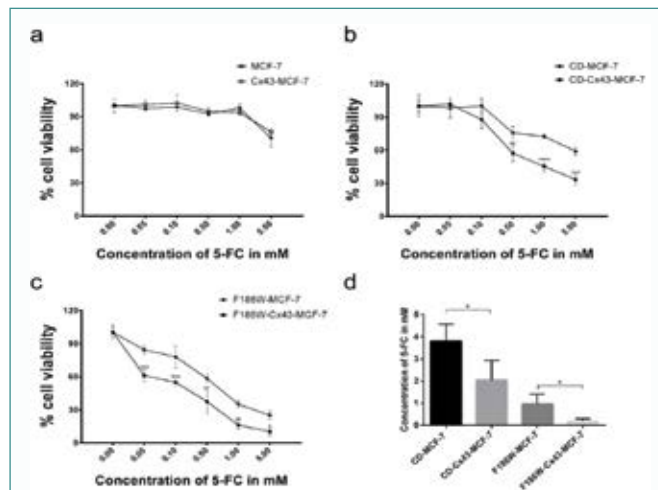


Figure 2: Dose-dependent cell viability assays. a) Reduction in cell viability was assessed after treating MCF-7 cells and Cx43-MCF-7 cells with different concentrations of 5-FC. CD-MCF-7 & CD-Cx43-MCF-7 cells. (b) and F186W-MCF-7 & F186W-Cx43-MCF-7 cells. c) Were subjected to 5-FC treatment for 72 hr. The reduction in cell viability was assessed using MTT assay. d) IC₅₀ value of MCF-7 and Cx43-MCF-7 transfected with CD/F186W mutant was calculated using non-linear curve regression analysis and the level of significance were calculated using unpaired two-tailedt-tests (n=3, p=0.0020).

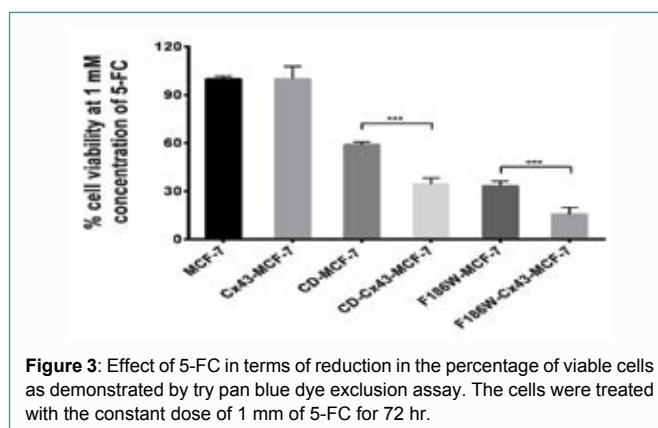


Figure 3: Effect of 5-FC in terms of reduction in the percentage of viable cells as demonstrated by try pan blue dye exclusion assay. The cells were treated with the constant dose of 1 mm of 5-FC for 72 hr.

Cx43-MCF-7 cells showed 31.79% of the apoptotic cell population. Whereas F186W-MCF-7 showed 39.05% of the apoptotic cell population and F186W-Cx43-MCF-7 cells showed 52.02% of the apoptotic cell population (Figure 4).

To further validate the data generated by the PI apoptotic assay, PE Annexin V and 7-AAD flow cytometric assay was performed (Figure 5). 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

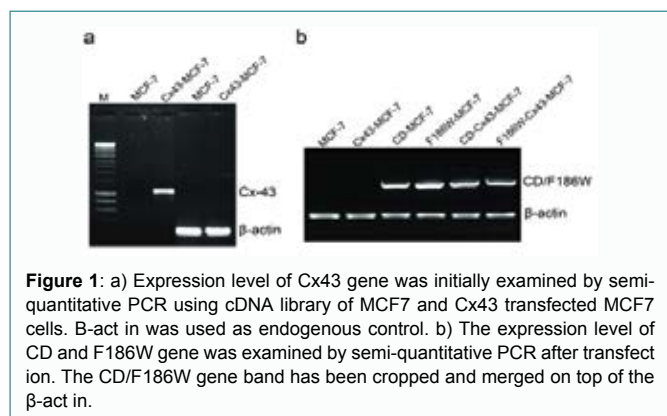


Figure 1: a) Expression level of Cx43 gene was initially examined by semi-quantitative PCR using cDNA library of MCF7 and Cx43 transfected MCF7 cells. B-act in was used as endogenous control. b) The expression level of CD and F186W gene was examined by semi-quantitative PCR after transfect ion. The CD/F186W gene band has been cropped and merged on top of the β -act in.

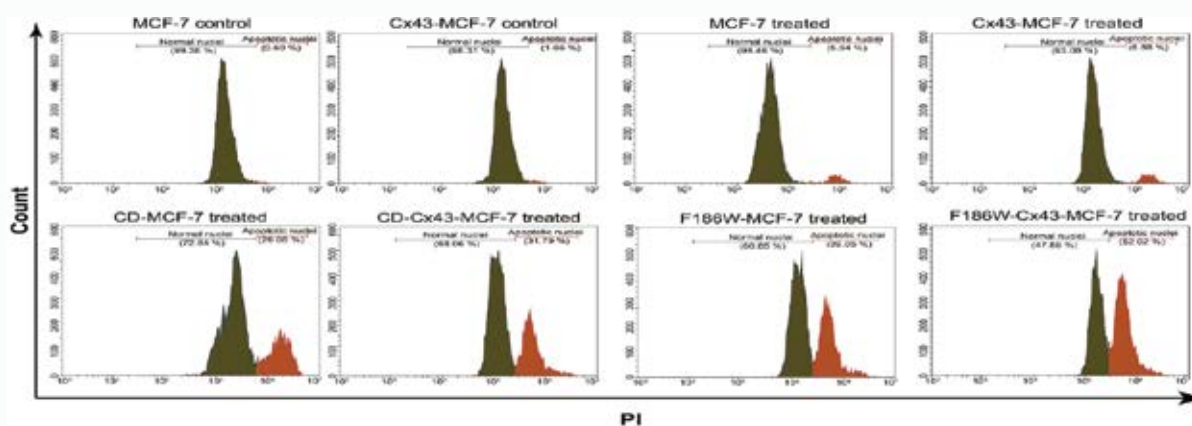


Figure 4: PI-based apoptotic cell analysis using flow cytometer.

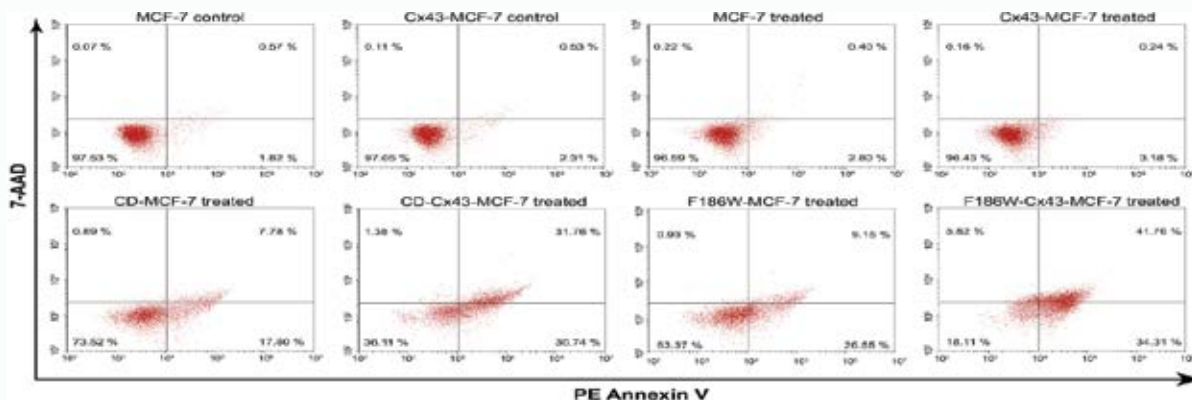


Figure 5: Assessment of early apoptotic, late apoptotic and necrotic cells was performed using PE Annexin V and 7-AAD based assay.

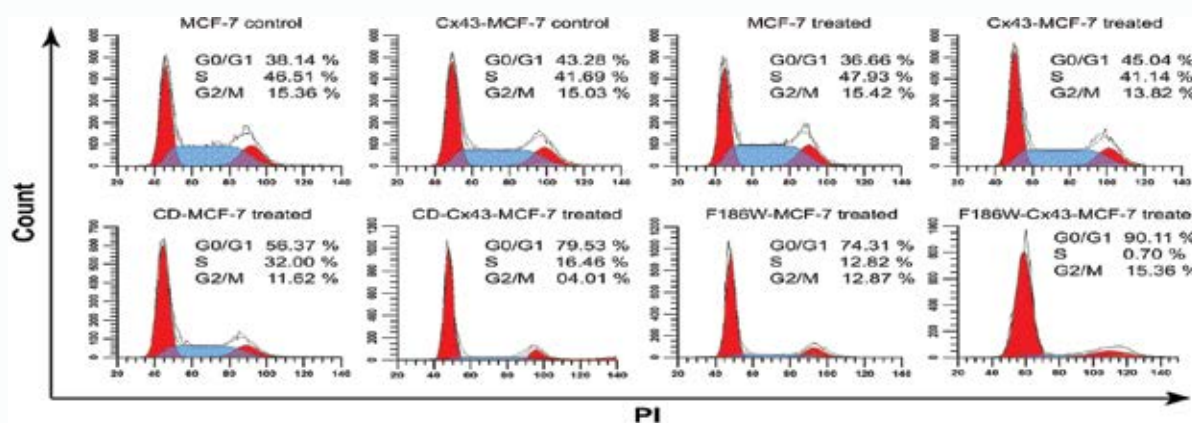


Figure 6: The synchronized cells were treated with 5-FC for 48 hr. At the end of the treatment duration, the phases of the cell cycle were evaluated using PI dye. The data were acquired in the flow cytometer and analyzed using ModFit LT software.

investigated by analysing the DNA content of the cells using PI. Figure 6 showed the flow cytometry data analyzed 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 [9]. 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%. Cx43 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 i.e. 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 affectivity 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% to 20% [10]. 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 [11-13]. The CD/5-FC gene transfer approach is based on the fact that 5-FU is generated from the pro drug 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 5-FU, around 20-fold lesser, when compared with that of its natural substrate cytosine [14]. In some cases, to reach a desirable response, the 5-FC doses have been increased, which led to the several side-effects [15]. To circumvent the dose dependent side-effects of the bacterial CD, a CD mutant F186W was designed in our laboratory [16]. The F186W mutant showed its superior therapeutic potency in A549 cells by demonstrating its high affinity towards 5-FU [7]. It was more efficient in converting the pro drug 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 suicide gene therapy 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 suicide gene therapy. 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 [17]. MCF-7, a human breast adenocarcinoma cell line, used in our study is devoid of Cx43 expression and functional GJIC [18]. The forced expression of Cx43 in MCF-7 cells made it susceptible to various chemotherapeutic treatments [19]. It was evident that Cx43 act as an anti-tumour protein by means of gap junction dependent as well as independent pathways [6]. Taken together, we presume that expression of Cx43 gene with the CD/F186W gene could make the MCF-7 cells more susceptible for the suicide gene therapy. 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 was 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. Cx43 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 delineated that the over expression of Cx43 in MCF7 cells could enhance the sensitivity of MCF-7 cells towards chemotherapeutic drugs underlying mechanism associated with it [3]. 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 antitumor protein Cx43 provided an excellent model to enhance the suicide gene therapy. 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 micro flora, the current 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) [9].

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Authors Contribution: 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 datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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