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Recombinant deoxyribonucleoside kinase from *Drosophila melanogaster* can improve gemcitabine based combined gene/chemotherapy for targeting cancer cells

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ABSTRACT

A recombinant deoxyribonucleoside kinase from *Drosophila melanogaster* with a deletion of the last 20 amino acid residues (named *Dm*dNKΔC20) was hypothesized as a potential therapeutic tool for gene therapy due to its broad substrate specificity and better catalytic efficiency towards nucleosides and nucleoside analogs. This study was designed to evaluate the effect of *Dm*dNKΔC20 for sensitizing human cancer cell lines to gemcitabine and to further investigate its role in reversal of acquired drug resistance in gemcitabine-resistant cancer cell line. The *DmdNKΔC20* gene was delivered to three different cancer cell lines, including breast, colon and liver cancer cells, using lipid-mediated transfection reagent. After transfection, gene expression of *DmdNKΔC20* was confirmed by quantitative reverse transcription PCR (qRT-PCR) and the combined effect of *Dm*dNKΔC20 and gemcitabine based cytotoxicity was observed by cell viability assay. We further evolved a gemcitabine-resistant breast cancer cell line (named MCF7-R) through directed evolution in the laboratory, which showed 375-fold more resistance compared with parental MCF7 cells. Upon transfection with *DmdNKΔC20* gene, MCF7-R cells showed 83-fold higher sensitivity to gemcitabine compared with the control group of MCF7-R cells. Moreover, we observed 79% higher expression of p21 protein in transfected MCF7-R cells, which may indicate induction of apoptosis. Our findings highlight the importance and therapeutic potential of *DmdNKΔC20* in combined gene/chemotherapy approach to target a wide range of cancers, particularly gemcitabine-resistant cancers.

KEY WORDS: *Drosophila melanogaster* deoxyribonucleoside kinase; suicide gene therapy; breast cancer; drug resistance; gemcitabine
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INTRODUCTION

Nucleoside analogs are being effectively used in clinics for the management of cancer, either alone or in combination with other drugs [1,2]. Gemcitabine, also known as 2', 2'-difluoro-deoxycytidine (dFdC), is a potent nucleoside analog used against a wide range of solid tumors [1,3-5]. Like other nucleoside analogs, gemcitabine is transported to the cells by human equilibrative and concentrative membrane transporters, where it is phosphorylated to the monophosphate form (dFdMP) by deoxycytidine kinase (dCK) [6]. dFdMP is further phosphorylated to cytotoxic diphosphate (dFdCDP) and triphosphate (dFdCTP) forms by intrinsic nucleotide kinases [7]. The diphosphorylated form of gemcitabine (dFdCDP) inhibits ribonucleotide reductase (RNR) causing a decrease in intracellular dCTP concentration. Consequently, increased phosphorylation of gemcitabine to its di and triphosphate forms occurs, leading to accelerated incorporation of dFdCTP into replicating DNA [8]. The triphosphorylated form of gemcitabine incorporates into DNA, thereby causing chain termination and apoptosis [5,9].

Akin to other nucleoside analog-based chemotherapies, the clinical significance of dFdC is limited by the development of drug resistance, either through intrinsic factors such as hypoxia, extracellular matrix transitions (EMT), and reactive oxygen species (ROS)-induced changes, or by acquiring alterations in drug activation and metabolic pathways [10-16]. dCK plays an important role in the activation of gemcitabine through phosphorylation, and an altered expression of dCK has been correlated with the development of gemcitabine resistance both *in vitro* and *in vivo* [17-21]. Besides dCK, several

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other targets of gemcitabine metabolism have been associated with acquired resistance to gemcitabine [22,23]. One of those targets is cytidine deaminase (CDA), for which increased expression was associated with decreased sensitivity of cancer cells to gemcitabine both *in vitro* and *in vivo*. CDA converts dFdC to difluorodeoxyuridine (dFdU), thus contributing to gemcitabine resistance in cancer cells [24].

Different therapeutic approaches have been proposed to overcome resistance due to nucleoside kinase deficiency, such as the introduction of monophosphate derivatives of the nucleoside analogs into the cells or nucleoside kinase enzymes by gene therapy [20,25-30]. Gene-directed enzyme prodrug therapy (GDEPT), also called suicide gene therapy, has been shown to be more effective for specific targeting of cancer cells as compared to standard chemotherapy [31]. Suicide gene therapy has been employed successfully in conjunction with human dCK [22-25,27,28,31,32]; however, a non-human dNK, known as Herpes simplex 1 thymidine kinase (HSV1-TK), has also served as an archetype for this approach [33,34].

The kinetic properties of the kinases used in GDEPT approach are one of the limiting factors, therefore, a better catalytic enzyme that could render more sensitivity to nucleoside analogs in cancer cells is desired. The landmark discovery of a multisubstrate nucleoside kinase from *Drosophila melanogaster* (called *DmdNK*), in 1998, ushered the kinases research into a new era. Based on its unique capacity to catalyze more efficiently the phosphorylation of all natural nucleosides and its substantial activity against a wide range of nucleoside analogs, *DmdNK* was distinguished among all nucleoside kinases for its potential use in suicide gene therapy [35,36]. The amino acid alignment data and structural knowledge of *Dm*dNK show its structural similarity with non-TK1 family of nucleoside kinases, which include mammalian TK2, dCK, and dGK [36,37]. Munch-Petersen et al. further studied recombinant *Dm*dNK and generated its C-terminus truncated versions called *Dm*dNKΔC10, *Dm*dNKΔC20, and *Dm*dNKΔC30 by deleting the last 10, 20, and 30 amino acid residues, respectively [37]. The deletion of the last 20 amino acid residues from the C-terminus increased the specific activity by 2-fold, whereas the deletion of the last 30 C-terminal residues resulted in an almost inactive enzyme. Furthermore, *Dm*dNKΔC20 was shown to be more stable than the wild-type or full-length *Dm*dNK [37].

*Dm*dNK and its improved variants, produced by site-directed mutagenesis, have been tested for their optimum application in suicide gene therapy [38-40]. Both viral (adeno- and lentivirus-based vectors) and non-viral delivery systems have been used for transfecting different cancer cells with *Dm*dNK and its mutants, to study the combined cytotoxic effect of gene/prodrug and to reverse drug resistance in various cancer cell lines [9,41-43]. To the best of our knowledge, *Dm*dNKΔC20 has not been reported neither for sensitizing human cancer cell lines to gemcitabine nor for the reversal of gemcitabine resistance in drug-resistant cancer cells so far. Therefore, we studied the effect of *Dm*dNKΔC20 to sensitize different human cancer cell lines to gemcitabine, and we further elaborated its role in the reversal of acquired gemcitabine resistance due to dCK deficiency in the breast cancer cell line MCF7-R.

MATERIALS AND METHODS

Chemicals

Gemcitabine, MTT (methylthiazolyldiphenyl-tetrazolium bromide), and all other standard chemicals were purchased from Sigma-Aldrich, USA unless mentioned otherwise. Cytarabine (araC) used in this study was obtained from Pfizer. GeneJet RNA purification kit (cat. # K0731), RevertAid first strand cDNA synthesis kit (cat. # K1622), and SYBR Green (cat. # K0221) were purchased from Thermo Fisher Scientific, USA. DMEM cell culture medium supplemented with Glutamax and sodium pyruvate (cat. # 1880272), fetal bovine serum (FBS), penicillin/streptomycin, Opti-MEM™ (cat. # 1758537), trypsin, and cell culture grade PBS were obtained from Gibco® (Thermo Fisher Scientific, USA). Lipofectamine 3000® was purchased from Invitrogen (Thermo Fisher Scientific, USA). Primary antibodies were obtained from Santa Cruz Biotechnology, USA as follows: dCK (sc-81245), CDA (sc-365292), p21 (sc-756), and β-actin (sc-130656). The following secondary antibodies were used from Invitrogen: goat anti-mouse IgG, horseradish peroxidase conjugate (Catalog No G21040) and goat anti-rabbit IgG, horseradish peroxidase conjugate (Catalog No G21234).

Cell culture

Breast cancer cell line MCF7, colon cancer cell line HCT116, and liver cancer cell line HePG2 were kindly provided by Professor Staffan Johansson (Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden). MCF7 cells resistant to 2 μM gemcitabine (named MCF7-R) were evolved by exposing wild-type MCF7 (MCF7-wt) to gradually increasing concentrations of gemcitabine over a period of 7 months. MCF7-R cells were viable in a medium containing 2 μM gemcitabine. The cancer cell lines used in this study were maintained in DMEM supplemented with 10% (v/v) FBS and penicillin/streptomycin (1%). Cells were grown in culture flasks with vented caps at 37°C in an incubator with 5% CO_2.

Cell viability assay

Cell viability was analyzed by standard MTT assay. Cells from respective cell lines were seeded in triplicate with a

seeding density of 5000–7500 cells per well in 100 µl of fresh culture medium in 96-well plates. After 24 hours of incubation at 37°C, the culture medium was replaced with 100 µl of fresh medium containing different concentrations of either araC or gemcitabine depending on the experiment. In the presence of the candidate drug, cells were further incubated for 72 hours at 37°C under 5% CO_2. After the incubation, 10 µl of MTT (5 mg/ml) was added and the cells were further incubated at 37°C for 4 hours. This resulted in the formation of formazan crystals due to mitochondrial dehydrogenase activity of viable cells; afterwards, the medium was replaced with 100 µl of acidified isopropanol (0.04N HCl in isopropanol) to dissolve the formazan crystals. The absorbance was recorded at 492 nm using Labtech LT-4500 plate reader. IC_{50} was defined as the drug concentration that reduces cell proliferation to 50% and calculated using equation 1. Survival curves were generated and analyzed by the non-linear regression function of GraphPad Prism 7 for Windows (GraphPad Software, La Jolla California USA). Resistance ratio was calculated as the ratio between IC_{50} of resistant cells to the IC_{50} of wild-type MCF7 cells.

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{(1+10\text{^}((\text{Log}\,IC_{50} - X)*\text{Hill Slope})))} \quad (1)$$

Transfection of cancer cells with *DmdNKΔC20*

DmdNKΔC20 cloned in pGEX-2T vector was kindly provided by the late Professor Jure Piskur (Department of Biology, Lund University, Sweden). *DmdNKΔC20* was restricted with BamH1 and EcoR1 and cloned into pcDNA3 vector. For transfection studies, 70–80% confluent cancer cells were used. The cancer cells were transfected with pcDNA3 vector containing *DmdNKΔC20* using Lipofectamine® 3000 reagent following the protocol described in Lipofectamine® 3000 reagent kit. For control groups, empty pcDNA3 vector was used for transfecting the cancer cells using the same protocol. The transfected cells were incubated overnight before seeding into 96-well plates for cell viability assay (described earlier). Moreover, the total mRNA and protein of both groups of transfected cells were isolated 72 hours after the transfection for subsequent quantitative reverse transcription PCR (qRT-PCR) and western blot analyses.

Quantitative reverse transcription PCR (qRT-PCR)

The total mRNA was extracted from cells using GeneJet RNA purification kit according to the manufacturer's protocol and quantified by NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). After DNase-I treatment of template, 1 µg of cDNA was synthesized using the protocol of RevertAid first strand cDNA synthesis kit and used as a template in qRT-PCR employing SYBR Green chemistry for the detection of quantified product. The reaction was performed in a thermal cycler (BioRad-CFX) with amplification profile as follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 58°C for 20 seconds, and 72°C for 20 seconds. The sequences of primers used for gene expression analysis in this study are provided in Table S1.

Western blot analysis

Total protein was extracted from transfected and control MCF7 groups using Laemmli method [44]. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane for detection. Ponceau S staining was performed to verify the successful blotting of proteins on the PVDF membrane. Membranes were blocked using 5% skimmed milk in TBST. After blocking, membranes were incubated with primary antibody against actin (1:250 dilution), dCK (1:50 dilution), CDA (1:1000 dilution), and p21 (1:500 dilution) and further incubated with HRP-conjugated secondary antibodies (1:10,000 dilution). Protein signals were detected by chemiluminescence and exposed to Kodak films for quantification of the intensity of detected bands.

Statistical analysis

Each experiment was performed in triplicate and the results are expressed as the mean ± standard error (SE). Statistical analyses including Student's *t*-test were performed using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp., Armonk, NY). Transfected and control groups were compared by paired *t*-test analysis using GraphPad Prism 7. Experiments with $p < 0.05$ were considered statistically significant and **** represents $p < 0.0001$.

RESULTS

Development and characterization of 2 µM gemcitabine-resistant cells

Gemcitabine-resistant MCF7 (MCF7-R) cells were evolved by exposing wild-type MCF7 cells to gradually increasing concentrations of gemcitabine over a period of 7 months. The resulting MCF7-R cells showed 375-fold higher resistance to gemcitabine compared with wild-type (MCF7-wt) cells. Moreover, MCF7-R cells showed a significant cross-resistance to araC with a resistance ratio of 39-fold. The gene and protein expressions of enzymes dCK and CDA were estimated by qRT-PCR and western blot analysis, respectively (Figure 1). An altered expression of both dCK and CDA was observed in MCF7-R cells in comparison to the wild-type counterpart. The gene expression of dCK was decreased to 2.7-fold in MCF7-R compared with wild-type MCF7, whereas the

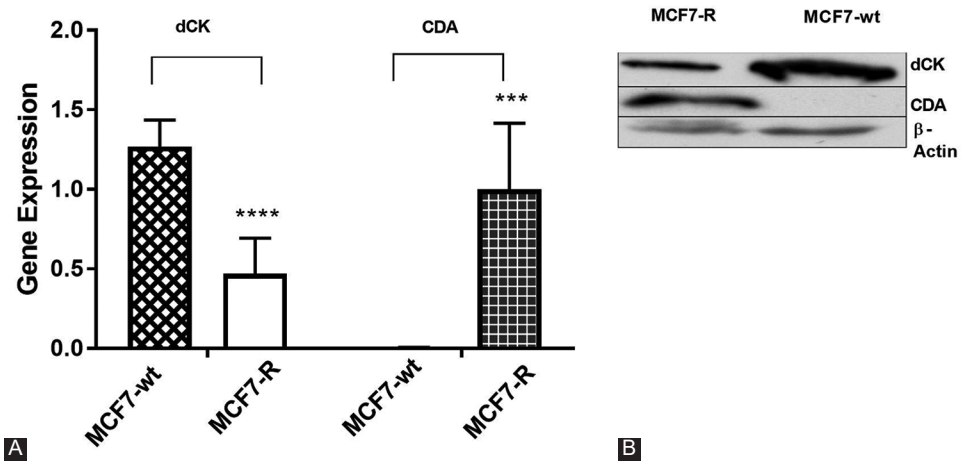

FIGURE 1. (A) Gene expression of deoxycytidine kinase (dCK) and cytidine deaminase (CDA) in MCF7-wt and MCF7-R cell line was measured by quantitative reverse transcription PCR (qRT-PCR). Error bars represent standard error (SE). Two-tailed *p* values obtained by *t*-test, **** is $p < 0.0001$, *** is $p < 0.001$. (B) Protein expression of dCK and CDA was quantified by western blotting in MCF7-wt and MCF7-R cells, ß-actin was used as an internal control. An altered expression of both dCK and CDA was observed in MCF7-R cells in comparison to the wild-type counterpart. The gene expression of dCK was decreased to 2.7-fold in MCF7-R compared with wild-type MCF7, whereas the expression of CDA was 187-fold higher in MCF7-R cells compared with near basal level expression of this enzyme in MCF7 wild-type.

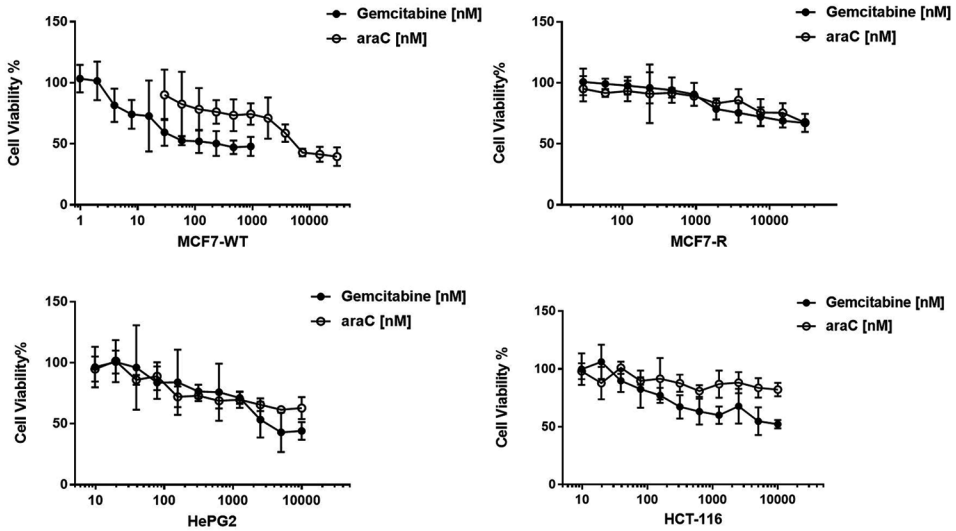

FIGURE 2. Cell viability of MCF7-wt, MCF7-R, HepG2, and HCT116 in the presence of gemcitabine and cytarabine (araC) was analyzed by MTT assay; each experiment was performed in triplicate. X-axis represents drug concentration and Y-axis represents cell survival in the presence of drug. Error bars represent standard error (SE).

expression of CDA was 187-fold higher in MCF7-R cells compared with near basal level expression of this enzyme in MCF7 wild-type. Beta-2 microglobulin (B2M) gene expression was used as an internal control to normalize the expression of dCK and CDA. Similar changes in protein expression of the two enzymes were also observed in western blot analysis.

Determination of IC_{50} for gemcitabine and araC before transfection of *DmdNKΔC20*

IC_{50} for gemcitabine and araC were determined by MTT assay for breast, colon and hepatic cancer cell lines. The survival curves were generated and analyzed by non-linear regression analysis using GraphPad Prism 7 (Figure 2). IC_{50} values for the cell lines determined in this study are shown in Table 1.

Effect of *Dm*dNKΔC20 on wild-type and gemcitabine-resistant human cancer cell lines

The combined cytotoxic effect of suicide gene (*DmdNKΔC20*) and gemcitabine was studied using breast cancer (MCF7-wt and MCF7-R), colon cancer (HCT116) and hepatic cancer (HepG2) cell lines. The qRT-PCR results confirmed the expression of *DmdNKΔC20* in transfected samples of each cell line. Human B2M expression was used as a reference for normalization (Figure 3).

MTT assay was performed to calculate the IC_{50} value for gemcitabine in each transfected cell line and compared with respective controls. Survival curves were generated (Figure 4) and data were analyzed by non-linear regression using GraphPad Prism 7.

A significant increase in the sensitivity of transfected cell lines to gemcitabine was observed, as shown by a decrease in IC_{50} compared with control group. The IC_{50} and folds of sensitivity of each transfected cell line for gemcitabine are described in Table 2.

TABLE 1. IC_{50} values (in μM) for nucleoside analog effect on different cancer cell lines before transfecting *DmdNKΔC20*

Cancer cell line	IC_{50} for gemcitabine	IC_{50} for araC	*p*
MCF7-wt	0.004±0.00071	3.5±0.0003	****
MCF7-R	1.5±0.00017	138±0.0092	****
HCT116	0.082±0.00039	0.1735±0.00039	****
HePG2	2.9±0.002	0.093±0.00029	****

All experiments are representative values of triplicate assay and results are expressed as mean values±SE. ****indicates statistically highly significant ($p<0.0001$), obtained by Student's *t*-test. ****represents statistically significant $p<0.0001$, for 2 μM gemcitabine-resistant MCF7 and wild-type MCF7 cells. SE: Standard error

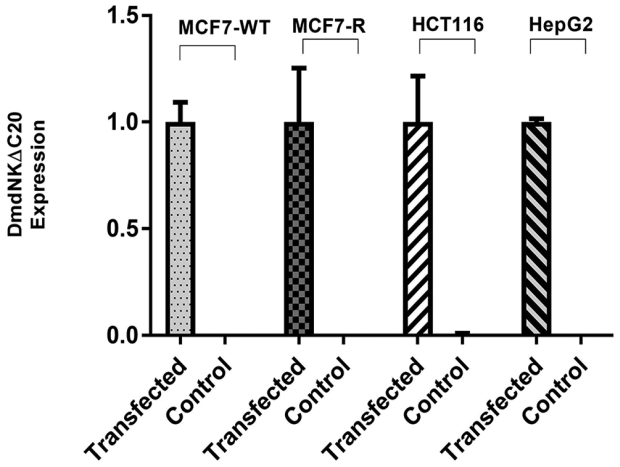

FIGURE 3. Expression of *DmdNKΔC20* in human cancer cell lines after transfection, measured by quantitative reverse transcription PCR (qRT-PCR). Error bars represent standard error (SE).

Furthermore, gemcitabine-resistant MCF7-R cells transfected with *DmdNKΔC20* became 83-fold more sensitive to gemcitabine than control, showing significant reversal of gemcitabine resistance upon transfection with *DmdNKΔC20*. A comparison of IC_{50} ratios of control and transfected MCF7-R cells provided extremely significant statistical results (paired *t*-test was performed as mentioned in Table 2).

Detection of p21 protein as apoptotic marker

Previous studies showed the role of p21 protein in growth arrest and induction of apoptosis by either p53 dependent or independent activation pathways and indicated that its upregulation in cancer cell lines is probably a consequence of anticancer treatment [45-48]. The western blot analysis using cell lysates showed 79% higher level of p21 protein expression in transfected MCF7-R cells compared with the control, which may indicate the induction of apoptosis as described earlier (Figure 5). The p21 specific primary antibody expression was normalized against ß-actin.

DISCUSSION

Gemcitabine is a potent nucleoside analog, which has been shown to have substantial anticancer activity against a wide range of solid tumors [5,9]. Due to its low toxicity profile, gemcitabine has also been recommended as an effective drug for use in suicide gene therapy [49]. The truncated variant

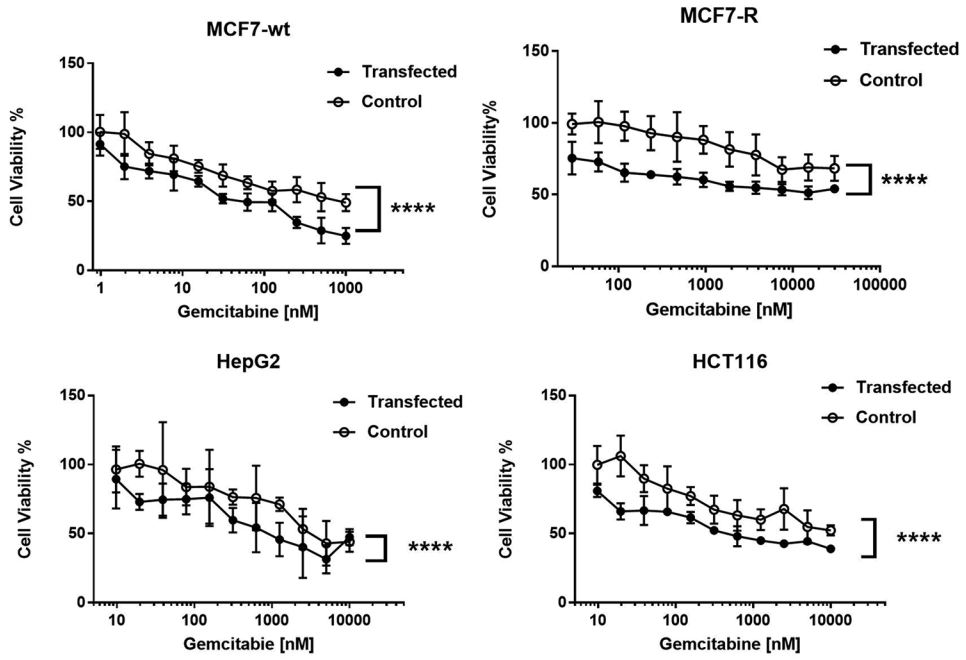

FIGURE 4. Cell viability of MCF7-wt, MCF7-R, HepG2, and HCT116 cancer cells after transfection with *DmdNKΔC20;* each experiment was performed in triplicate. Error bars represent standard error (SE). The results were further analyzed by Prism's paired *t*-t est analysis. **** represents $p < 0.0001$. A significant increase in the sensitivity of transfected cell lines to gemcitabine upon transfection of *DmdNKΔC20* was observed, as shown by a decrease in IC_{50} compared with control group.

TABLE 2. IC_{50} values (in μM) and increase folds in the sensitivity to gemcitabine of each cell line transfected with either empty vector (control) or with *DmdNKΔC20*

Cell line	Control	Transfected with *DmdNKΔC20*	Sensitivity folds	*p*
MCF7-wt	0.004±0.00071	0.000015±0.0000011	267	****
MCF7-R	1.5±0.0003538	0.018±0.005	83.3	****
HCT116	0.082±0.0003915	0.0088±0.0004	9.3	****
HePG2	2.9±0.002	0.391±0.0002	7.4	****

All experiments were performed in triplicate and results are expressed as mean values±SE. Sensitivity folds were calculated by comparing the IC_{50} of transfected and control cells. ****Represents statistically significant $p<0.0001$, obtained by paired *t*-test. The *DmdNKΔC20* transfected cells were compared to those cells where empty vector was used for transfection (named control). SE: Standard error

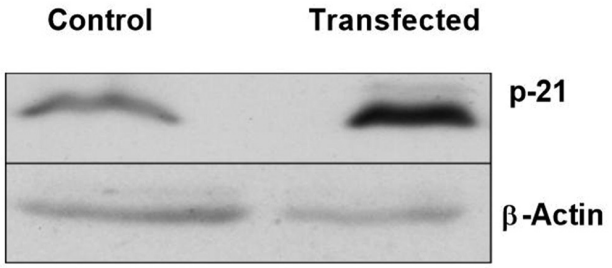

FIGURE 5. Western blot was performed on *DmdNKΔC20* transfected and control MCF7-R cells grown in the presence of 2 μM gemcitabine for 72 hours. The p-21 protein was highly expressed in transfected MCF7-R cells as compared to control, which possibly indicates the induction of apoptosis as a result of transfection.

of *Drosophila* nucleoside kinase, called *DmdNKΔC20*, has been reported to be highly efficient for the phosphorylation of gemcitabine, and its crystal structure bound with gemcitabine further supports this character [40]. Knecht et al. have previously shown 44- and 4-fold reversal of gemcitabine resistance in ovarian cancer cell line (AG6000) and glioblastoma cell line (U-87 MG), respectively, upon transduction with a full-length *Dm*dNK [40]. In this study, we explored the efficacy of *DmdNKΔC20* to sensitize different cancer cell lines to gemcitabine *in vitro* and we further studied the reversal of gemcitabine resistance in the breast cancer cell line MCF7-R upon transfection with *DmdNKΔC20*.

In line with our hypothesis and the results from earlier studies, a significant increase in the sensitivity to gemcitabine was observed in the wild-type MCF7, HCT116 and HepG2 cancer cell lines transfected with *DmdNKΔC20*, as a result of the combined enzyme/prodrug effect (Table 2). Moreover, due to the expression of *DmdNKΔC20*, the gemcitabine-resistant cancer cells MCF7-R exhibited 83-fold increase in sensitivity to gemcitabine when compared with the corresponding control (Table 2). MCF7-R cells showed lower dCK and higher *CDA* expression which favored and conferred higher gemcitabine resistance in the evolved MCF7-R cells. However, after transfection with *DmdNKΔC20*, MCF7-R cells appeared to become more sensitive to gemcitabine by losing their drug resistance phenotype. The observed effect is considerably higher than previously reported for human uterine sarcoma cell line Messa 10K, where a full-length *DmdNK* was transfected to sensitize human cancer cell lines to various nucleosides [9,41-43, 50-52]. A possible explanation for the increased sensitivity to gemcitabine is the lower K_m value of *DmdNKΔC20* for this analog and unique interaction of fluoride atoms of gemcitabine [40]. The strong binding affinity of *DmdNKΔC20* towards gemcitabine enhances the phosphorylation of gemcitabine that may result in an increased apoptosis with high p21 protein expression in transfected cells, as observed in Figure 5.

The results shown in this study are comparable to previously reported results in which a full-length *Dm*dNK with different nucleoside analogs has been used [5,9,20,26,27,53-55]. However, our findings emphasize the role of the truncated version of *Drosophila melanogaster* enzyme, *Dm*dNKΔC20, in sensitizing different cancer cells to gemcitabine and elaborate its role in the reversal of gemcitabine resistance in MCF7 cancer cells *in vitro*. These findings may be useful for future studies aiming to explore the potential use of *Dm*dNKΔC20 in combined enzyme/prodrug therapeutic approach in treating a wide range of cancers, particularly gemcitabine-resistant cancers.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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SUPPLEMENTAL DATA

TABLE S1. Primers used in qRT-PCR experiments

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
dCK	TCCATCGAAGGGAACATCGC	CATCTGGCAACAGGTTCAGGA
CDA	TCGCCAGTGACATGCAAGAT	CCATCCGGCTTGGTCATGTA
*Dm*dNKΔC20	TGACCATGCTGCAGTCGCACA	CGCATGTTCTCCACGAAGCAA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

dCK: Deoxycytidine kinase; CDA: Cytidine deaminase; B2M: Beta-2 microglobulin; qRT-PCR: Quantitative reverse transcription polymerase chain reaction