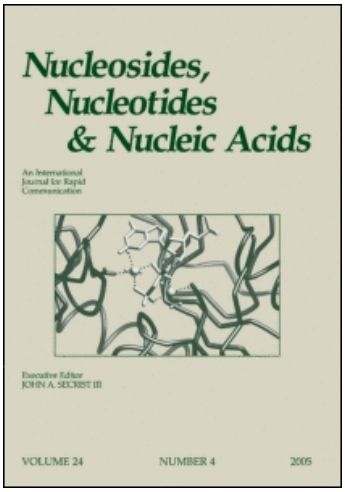

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Transient Expression of *Drosophila melanogaster* Deoxynucleoside Kinase Gene Enhances Cytotoxicity of Nucleoside Analogs

Hiroyuki Kamiya[ab]; Hiroshi Ochiai[ab]; Hideyoshi Harashima[ab]; Mana Ito[a]; Akira Matsuda[a]
[a] Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan [b] CREST, Japan Science and Technology, Japan

To cite this Article Kamiya, Hiroyuki , Ochiai, Hiroshi , Harashima, Hideyoshi , Ito, Mana and Matsuda, Akira(2006) 'Transient Expression of *Drosophila melanogaster* Deoxynucleoside Kinase Gene Enhances Cytotoxicity of Nucleoside Analogs', Nucleosides, Nucleotides and Nucleic Acids, 25: 4, 553 — 560
To link to this Article: DOI: 10.1080/15257770600685784
URL: http://dx.doi.org/10.1080/15257770600685784

Nucleosides, Nucleotides, and Nucleic Acids, 25:553–560, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600685784

Taylor & Francis
Taylor & Francis Group

TRANSIENT EXPRESSION OF *Drosophila melanogaster* DEOXYNUCLEOSIDE KINASE GENE ENHANCES CYTOTOXICITY OF NUCLEOSIDE ANALOGS

Hiroyuki Kamiya, Hiroshi Ochiai, and Hideyoshi Harashima □ *Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan, and CREST, Japan Science and Technology, Japan*

Mana Ito and Akira Matsuda □ *Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan*

□ *The* Drosophila melanogaster *deoxynucleoside kinase gene was introduced into HeLa cells with cationic lipids to allow its transient expression, and cytotoxic effects of several nucleoside analogs in the transfected cells were examined. Of the analogs tested, cytotoxicities of 1-β-D-arabinofuranosylcytosine (araC), 5-fluorodeoxyuridine (FUdR), and 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC) were increased by the deoxynucleoside kinase gene. These results suggest that the combination of the transient expression of the* Drosophila *deoxynucleoside kinase gene and these nucleoside analogs is a candidate for the suicide gene therapy.*

Keywords Deoxynucleoside kinase; Nonviral vector; Nucleoside analog; Suicide gene therapy

INTRODUCTION

The nucleotide metabolism is very important for living cells and is a target of many nucleoside analogs. In general, the nucleoside analogs are required to be phosphorylated in mammalian cells by nucleoside kinases. Deoxycytidine kinase, thymidine kinase-1, adenosine kinase, and uridine/cytidine kinase are present in the cytosol, and thymidine kinase-2 and deoxyguanosine kinase exist in mitochondri.[1–3] The phosphorylation of nucleoside analogs by these cellular kinases is thought to be a determinant factor for their efficacy.[4] To achieve their efficient phosphorylation, transfer of sui-

Received 1 January 2006; accepted 30 January 2006.
This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.
Address correspondence to Hiroyuki Kamiya, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan. E-mail: hirokam@pharm.hokudai.ac.jp

cide genes such as the Herpes simplex virus thymidine kinase gene has been examined. Administration of ganciclovir after introduction of the thymidine kinase gene has been shown to exert cytotoxic effects.[5–9]

Another candidate for the suicide gene used in the chemo/gene therapy is the *Drosophila melanogaster* deoxynucleoside kinase (Dm-dNK) gene. The Dm-dNK protein is a multisubstrate enzyme that phosphorylates the four natural pyrimidine and purine deoxyribonucleosides, and some nucleoside analogs.[10,11] In addition, the catalytic rate of deoxyribonucleoside phosphorylation by the Dm-dNK protein is 10- to 100-fold higher than any of the mammalian deoxyribonucleoside kinases. Thus, use of Dm-dNK is attractive in the chemo/gene therapy due to the broad substrate specificity and efficient catalytic rate. Previously, retroviral transduction of the Dm-dNK gene was conducted and cells with the gene became sensitive to some nucleoside analogs.[12,13] However, it is known that viral transduction causes severe side effects. Direct introduction of the Dm-dNK protein by liposomes was also examined and enhanced sensitivity to some nucleoside analogs was reported.[14] The direct delivery of the protein, however, is generally less efficient than DNA transfection.[15]

We focused on nonviral delivery of the Dm-dNK gene because nonviral vectors are excellently safe in comparison to viral vectors.[16–19] Although nonviral vectors are thought to be less efficient than viral vectors, protein production from DNA nonvirally delivered into dividing cells is efficient. At least a 10^3- to 10^5-fold molar amounts of protein is produced from one copy of the plasmid DNA molecule.[15] Thus, it is of importance to examine effects of the Dm-dNK gene introduction with nonviral vector on the sensitivity to nucleoside analogs, as a model of human chemo/gene therapy. In this study, we introduced the Dm-dNK gene into HeLa cells and treated the cells with seven nucleoside analogs. Three analogs were more toxic in the transfected cells than in the control cells, suggesting that nonviral delivery of the gene in combination with nucleoside analogs is useful as the suicide gene therapy.

MATERIALS AND METHODS

Construction of Plasmid DNA Carrying the Dm-dNK Gene

The Dm-dNK gene was cloned by the two step-PCR performed with high-fidelity Pyrobest DNA polymerase (Takara, Otsu, Japan) and inserted into the pGEX-6P-3 plasmid (Amersham Biosciences, Piscataway, New Jersey), to produce the pGST-dNK plasmid containing a glutathione-*S*-transferase-Dm-dNK fusion gene under the control of the *Escherichia coli tac* promoter. The construction of pGST-dNK will be reported elsewhere. To insert the Dm-dNK gene into a mammalian expression vector, the Kozac sequence and an *Sac*II site, and an *Xba*I site were introduced into the upstream and downstream of the gene, respectively, in the high-fidelity PCR using Pyrobest DNA

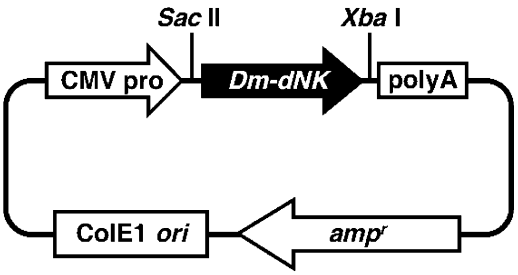

FIGURE 1 Structure of plasmid DNA containing the Dm-dNK gene and the CMV promoter used in this study. CMV pro, CMV promoter; *amp*^{r}, *E. coli* ampicillin resistance gene; *ori*, *E. coli* replication origin. *Sac*II and *Xba*I sites used for the construction are also shown.

polymerase and an upper primer (5′-GGCCCGCGGCCGCC***ATG***GCGGA where the *Sac*II site and initiation codon are underlined and italicized, respectively) and a lower primer (5′-CGCTCGTCTAGACTAATGGGATAA where the *Xba*I site is underlined). The amplified PCR fragment was digested with *Sac*II and *Xba*I, and ligated into the backbone part (the *Sac*II–*Xba*I large fragment) of the pYK-CMV-luc[20] to yield the plasmid DNA carrying Dm-dNK gene, pCMV-dNK (Figure 1).

This plasmid DNA was amplified in the *E. coli* strain DH5α and purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Maxi kit.

Nucleoside Analogs

1-β-D-Arabinofuranosylcytosine (araC), 3′-azido-3′-deoxythymidine (AZT), 2-chloro-2′-deoxyadenosine (CdA), 5-fluoro-2′-deoxyuridine (FUdR), and tubercidin were purchased from Sigma-Aldrich (St. Louis, Missouri). 1-(2-Deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC) was synthesized as described previousl.[21] Oxetanocin A (OXT-A) was a generous gift from Nippon Kayaku.

DNA Transfection

DNA transfection was carried out with the Lipofectamine Plus Reagent (Invitrogen, Carlsbad, California) essentially according to the supplier's instructions. HeLa cells (5×10^4 cells/well) were incubated in DMEM medium with 10% fetal calf serum under 5% CO_2/air at 37°C for 24 h. The pCMV-dNK plasmid (330 ng, 70 fmol) was mixed with "carrier DNA," the pBR322 plasmid, to give a total amount of 500 ng. The DNA was mixed with lipids and transfected into the cells. After 3 h, the lipid-DNA complex was removed, and the cells were incubated in DMEM with 10% fetal calf serum. After a further 21 h, the transfected cells were trypsinized and collected. Three thousand cells were seeded into a well and incubated in DMEM with 10% fetal calf serum containing a nucleoside analog for 48 h. Survival ratio was determined

with TetraColor One (Seikagaku, Tokyo, Japan) according to the supplier's instructions.

Detection of Dm-dNK mRNA by RT-PCR

Total RNA was extracted from HeLa cells transfected with the pCMV-dNK plasmid using an RNeasy Mini Kit combined with RNase-free DNase Digest Set (Qiagen) for the degradation of DNA in total RNA samples. First-strand cDNA synthesis was performed on 500 ng of total RNA using an oligo dT primer and an RNA PCR Kit (AMV) (Takara) as described by the manufacturer's instructions. Each of the mRNA transcripts was amplified by PCR using the following primers: dNK (+), 5′-CGCTCCATTTTTAGCGCTCGCTATTGCTTCGTGGAGAACA); dNK (−), (5′-GCATCGAGGACTAGGACCTTGCACGACTGCGGTCG). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was also detected by RT-PCR.

RESULTS AND DISCUSSION

The Dm-dNK gene delivered by retrovirus enhances cytotoxicities of some nucleoside analogs.[12,13] In this study, the Dm-dNK gene was delivered by cationic lipids because nonviral vectors do not cause severe side effects, and transient expression of the gene is sufficient to kill tumor cells. We introduced the Dm-dNK gene on the pCMV-dNK plasmid (Figure 1) into human tumor-derived HeLa cells by cationic lipid-mediated transfection to mimic transient DNA introduction into tumor cells with nonviral vector. The Dm-dNK gene is transcribed under the control of the CMV promoter, one of the strongest promoters in mammalian cells (Figure 1). The transfected HeLa cells were then treated with seven nucleoside analogs (Figure 2). HeLa cells transfected with the plasmid carrying the CMV promoter and the luciferase gene was used as the control.

The Dm-dNK and luciferase plasmid DNAs (70 fmol) were transfected into HeLa cells with cationic lipids. The transfected cells were incubated for 24 h to allow them to express the exogenous genes. The expression of the Dm-dNK gene was confirmed at the transcript level by RT-PCR (Figure 3). Under similar transfection conditions, 30–40% of cells expressed GFP (green fluorescence protein) (data not shown). The transfected cells were then treated with various concentrations of nucleoside analogs for 48 h. Survival ratio was determined with the formazan assay. The expression of these exogenous genes was not toxic under our experimental conditions (data not shown).

Table 1 shows the IC_{50} values of the nucleosides in the luciferase and Dm-dNK gene-transfected HeLa cells. The most cytotoxic nucleoside was tubercidin. Its IC_{50} values in the luciferase and Dm-dNK gene-transfected

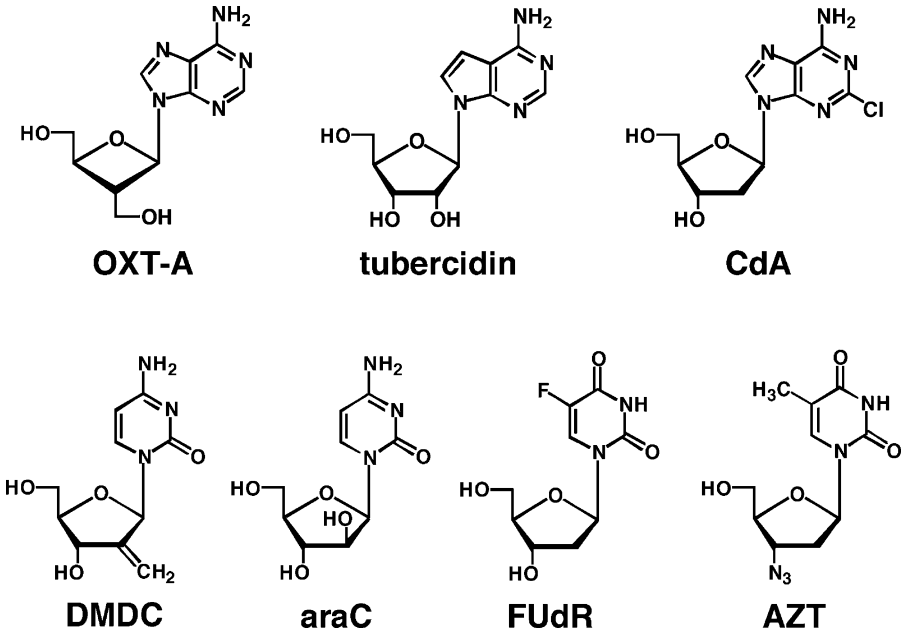

FIGURE 2 Structure of seven nucleoside analogs used in this study.

cells were ~0.1 μM. However, increase in the sensitivity by the introduction of the Dm-dNK gene was not clearly observed. In the case of araC, the IC_{50} values in the luciferase and Dm-dNK gene-transfected cells were 73 and 2 μM, respectively. The expression of the Dm-dNK gene enhanced its cytotoxicity by ~40-fold. The IC_{50} values of FUdR and DMDC in the Dm-dNK gene-transfected cells were below 10 μM. In particular, DMDC may be hopeful because its IC_{50} values were more than 100 μM for the control cells and ~8 μM for the Dm-dNK gene-transfected cells.

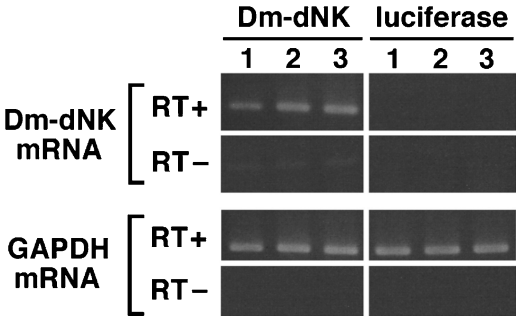

FIGURE 3 Expression of the Dm-dNK mRNA in the transfected HeLa cells. HeLa cells were transfected with the pCMV-dNK (Dm-dNK) or pYK-CMV-luc (luciferase) plasmid. After 24 h, total RNA was extracted and RT was carried out as described in the Materials and Methods section. The PCR products derived from mRNAs were visualized by staining with ethidium bromide of an agarose gel. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE 1 IC_{50} Values (μM) of Nucleosides in Transfected HeLa Cells

	Transgene		
Nucleoside	Luciferase	Dm-dNK	Ratio[a]
Tubercidin	0.14	0.092	1.5
araC	73	2.0	37
FUdR	83	8.7	9.5
DMDC	>100	7.8	>13
CdA	52	21	2.5
OXT-A	>100	>100	NA[b]
AZT	>100	>100	NA[b]

[a] The ratio of IC_{50} values in luciferase- to Dm-dNK-transfected HeLa cells.
[b] Not applicable.

The Dm-dNK gene was previously transduced by retroviral vector and increased sensitivities of some nucleoside analogs in the transduced cells were reported.[12] For thymidine kinase-1-deficient osteosarcoma cells, the efficacies of araC, CdA, and FUdR were 50- to 200-fold enhanced in the transduced cells. In thymidine kinase-1-proficient MIA-PaCa cells, however, the enhancement was observed only for CdA (~6-fold). In this study, the transient expression of the Dm-dNK gene in HeLa cells could increase sensitivities of araC and FUdR by more than 10-fold, but the efficacy of CdA was only slightly changed (Table 1). This discrepancy could be explained by difference in basal kinase activity and/or intracellular concentration of these analogs. Combinations of AZT, tubercidin, DMDC and OXT-A, and the Dm-dNK gene were first examined in this study, and DMDC was found to be more effective in the Dm-dNK gene-transfected cells. One of the reasons for more effective cytotoxicities of araC, FUdR, and DMDC in the transfected cells would be that they are good substrates for Dm-dNK. This interpretation, however, requires *in vitro* phosphorylation assay using purified Dm-dNK.

When we consider the *in vivo* application of the combination of the Dm-dNK gene and nucleoside analogs, tumor-specific expression of the Dm-dNK protein would be necessary. To accomplish this, tumor-specific delivery of the gene and use of a tumor-specific promoter would be required. We previously reported that carrier with ligand molecules for tumor-targeting, transferrin, was incorporated by the receptor-mediated endocytosis into cultured tumor cells very efficiently.[22] In addition, display of the GALA peptide on the surface of the carrier helped endosomal escape of the content.[22] The carrier with transferrin and GALA indeed expressed a marker gene in tumor cells *in vitro* (Sasaki et al., unpublished results). Thus, tumor-specific expression of the Dm-dNK gene would be achieved by the carriers as described above and tumor-specific promoters.

Another possible application of the combination of the Dm-dNK gene and nucleoside analogs is antiviral therapies. When binding site(s) of transcription factor(s) that positively regulate viral promoter(s) are introduced

onto the Dm-dNK plasmid, the cytotoxic action of a nucleoside analog would work specifically in infected cell.[23]

IC_{50} values of nucleoside analogs are affected by various factors except for the mono-phosphorylation efficiency. In particular, expressions of transporters concerning the influx and efflux of nucleoside analogs would be important and different cell by cell. It would be effective that gene(s) that regulate the influx or efflux of nucleoside analogs, as a gene for the influx transporter or a gene encoding siRNA for the efflux transporter, is introduced into tumor cells together with the Dm-dNK gene. Degradation enzymes such as cytidine deaminase also affect intracellular concentration of nucleoside analogs. Co-introduction of a gene encoding siRNA for the degradation enzyme would enhance effects of the Dm-dNK gene. DNAs for complete knock-out instead of the genes encoding siRNA might be used.[24]

In this study, we found that the transient expression of the Dm-dNK gene increased sensitivities of some nucleoside analogs. To further enhance their efficacies, more effective expression of the gene would be required. Improved DNA delivery systems and controlled intranuclear disposition[25] will help to produce more efficient nucleoside analog-phosphorylation systems with clinical applications.

REFERENCES

1. Arner, E.S.; Eriksson, S. Mammalian deoxyribonucleoside kinases. Pharmacology & Therapeutics **1995**, 67, 155–186.
2. Eriksson, S.; Munch-Petersen, B.; Johansson, K.; Eklund, H. Structure and function of cellular deoxyribonucleoside kinases. Cellular & Molecular Life Science **2002**, 59, 1327–1346.
3. Johansson, N.G.; Eriksson, S. Structure-activity relationships for phosphorylation of nucleoside analogs to monophosphates by nucleoside kinases. Acta Biochimica Polonica **1996**, 43, 143–160.
4. Matsuda, A.; Sasaki, T. Antitumor activity of sugar-modified cytosine nucleosides. Cancer Science **2004**, 95, 105–111.
5. Culver, K.W.; Ram, Z.; Wallbridge, S.; Ishii, H.; Oldfield, E.H.; Blaese, R.M. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science **1992**, 256, 1550–1552.
6. Tanaka, T.; Kanai, F.; Lan, K.H.; Ohashi, M.; Shiratori, Y.; Yoshida, Y.; Hamada, H.; Omata, M. Adenovirus-mediated gene therapy of gastric carcinoma using cancer-specific gene expression in vivo. Biochemical and Biophysical Research Communications **1997**, 231, 775–779.
7. Guo, S.Y.; Gu, Q.L.; Zhu, Z.G.; Hong, H.Q.; Lin, Y.Z. TK gene combined with mIL-2 and mGM-CSF genes in treatment of gastric cancer. World Journal of Gastroenterology **2003**, 9, 233–237.
8. Yamazaki, M.; Straus, F.H.; Messina, M.; Robinson, B.G.; Takeda, T.; Hashizume, K.; DeGroot, L.J. Adenovirus-mediated tumor-specific combined gene therapy using herpes simplex virus thymidine/ganciclovir system and murine interleukin-12 induces effective antitumor activity against medullary thyroid carcinoma. Cancer Gene Therapy **2004**, 11, 8–15.
9. Sieger, S.; Jiang, S.; Kleinschmidt, J.; Eskerski, H.; Schonsiegel, F.; Altmann, A.; Mier, W.; Haberkorn, U. Tumor-specific gene expression using regulatory elements of the glucose transporter isoform 1 gene. Cancer Gene Therapy **2004**, 11, 41–51.
10. Munch-Petersen, B.; Piskur, J.; Sondergaard, L. Four deoxynucleoside kinase activities from drosophila melanogaster are enzyme, a new multifunctional deoxynucleoside kinase. Journal of Biological Chemisty **1998**, 273, 3926–3931.
11. Johansson, M.; van Rompay, A.R.; Degreve, B.; Balzarini, J.; Karlsson, A. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of drosophila melanogaster. Journal of Biological Chemistry **1999**, 274, 23814–23819.

12. Zheng, X.; Johansson, M.; Karlsson, A. Retroviral transduction of cancer cell lines with the gene encoding drosophila melanogaster multisubstrate deoxyribonucleoside kinase. Journal of Biological Chemistry **2000**, 275, 39125–39129.
13. Zheng, X.; Johansson, M.; Karlsson, A. Nucleoside analog cytotoxicity and bystander cell killing of cancer cells expressing *Drosophila melanogaster* deoxyribonucleoside kinase in the nucleus or cytosol. Biochemical and Biophysical Research Communications **2001**, 289, 229–233.
14. Zheng, X.; Lundberg, M.; Karlsson, A.; Johansson, M. Lipid-mediated protein delivery of suicide nucleoside kinases. Cancer Research **2003**, 63, 6909–6913.
15. Yamada, Y.; Kamiya, H.; Harashima, H. Kinetic analysis of protein production after DNA transfection. International Journal of Pharmaceutics **2005**, 299, 34–40.
16. Mahato, R.I.; Takakura, Y.; Hashida, M. Nonviral vectors for In vivo gene delivery: physicochemical and pharmacokinetic considerations. Critical Reviews in Therapeutic Drug Carrier Systems **1997**, 14, 133–172.
17. Rolland, A.P. From genes to gene medicines: recent advances in nonviral gene delivery. Critical Reviews in Therapeutic Drug Carrier Systems **1998**, 15, 143–198.
18. Kamiya, H.; Tsuchiya, H.; Yamazaki, J.; Harashima, H. Intracellular trafficking and transgene expression of viral and non-viral gene vectors. Advanced Drug Delivery Reviews **2001**, 52, 153–164.
19. Niidome, T.; Huang, L. Gene therapy progress and prospects: nonviral vectors. Gene Therapy **2002**, 9, 1647–1652.
20. Ochiai, H.; Harashima, H.; Kamiya, H. Intranuclear disposition of exogenous DNA In vivo: silencing, methylation and fragmentation. FEBS Letters **2006**, 580, 918–922.
21. Matsuda, A.; Takenuki, K.; Tanaka, M.; Sasaki, T.; Ueda, T. Nucleosides and nucleotides. 97. Synthesis of new broad spectrum antineoplastic nucleosides, 2′-deoxy-2′-methylidenecytidine (DMDC) and its derivatives. Journal of Medicinal Chemistry **1991**, 34, 812–819.
22. Kakudo, T.; Chaki, S.; Futaki, S.; Nakase, I.; Akaji, K.; Kawakami, T.; Maruyama, K.; Kamiya, H.; Harashima, H. Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. Biochemistry **2004**, 43, 5618–5628.
23. Kovesdi, I.; Reichel, R.; Nevins, J.R. Identification of a cellular transcription factor involved in E1A trans-activation, Cell **1986**, 45, 219–228.
24. Tsuchiya, H.; Harashima, H.; Kamiya, H. Increased SFHR gene correction efficiency with sense single-stranded DNA. The Journal of Gene Medicine **2005**, 7, 486–493.
25. Kamiya, H.; Akita, H.; Harashima, H. Pharmacokinetic and pharmacodynamic considerations in gene therapy. Drug Discovery Today **2003**, 8, 990–996.