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Bystander Effects of Cancer Cell Lines Transduced with the Multisubstrate Deoxyribonucleoside Kinase of *Drosophila melanogaster* and Synergistic Enhancement by Hydroxyurea

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ABSTRACT

The multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster* (*Dm*-dNK) can be expressed in human cells with retained enzymatic activity. The cells expressing *Dm*-dNK exhibit increased sensitivity to several cytotoxic nucleoside analogs. In this study, we further evaluated *Dm*-dNK as a potential novel suicide gene in combination with (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) as the prodrug. We used two human cancer cell lines transduced with a retrovirus encoding the *Dm*-dNK cDNA and investigated whether the cells expressing the enzyme can induce cell death of untransduced cells, a phenomenon known as the "bystander effect". A bystander effect was observed in a thymidine kinase-deficient human osteosarcoma cell line but not in the MIA PaCa-2 human pancreatic adenocarcinoma cell line. The cytotoxicity of BVDU increased in both cell lines when the compound was used in combination with subtoxic concentrations of hydroxyurea. Hydroxyurea also enhanced the bystander effect in the osteosarcoma cells, but not in the MIA PaCa-2 cells, treated with BVDU. These findings indicate that BVDU phosphorylated by *Dm*-dNK in transduced cancer cells may also induce bystander cell death in certain cell lines.

The principle of suicide gene therapy is the transduction of cells with a gene that encodes an enzyme that can convert an inactive prodrug into a cytotoxic metabolite (Lal et al., 2000). A commonly used suicide gene approach involves the transfer of the herpes simplex virus type-1 thymidine kinase (HSV-1 TK) gene into malignant cells and subsequent treatment with ganciclovir (GCV) (Balzarini et al., 1985; Moolten, 1986; Moolten and Wells, 1990; Culver et al., 1992; Ram et al., 1997; Klatzmann et al., 1998). The HSV-1 TK-expressing tumor cells phosphorylate GCV to its cytotoxic triphosphate derivative, which interferes with DNA replication (Reardon, 1989) and induces cell death, probably by apoptosis (Freeman et al., 1993; Beltinger et al., 1999). In addition to affecting cells expressing HSV-1 TK, adjacent untransduced cancer cells are killed by the transfer of the phosphorylated nucleoside analog between cells (Freeman et al., 1993; Mesnil et al., 1996). This phenomenon, known as the "bystander effect", results in the killing of a larger portion of cells than is transduced with the suicide gene. The mechanism of the bystander effect is not fully understood. The uptake of apoptotic vesicles released from dying cells by adjacent nontransduced cells (Freeman et al., 1993) and the passage of phosphorylated GCV via gap junctions (Rubsam et al., 1999; Boucher et al., 2000) have been proposed as possible mechanisms.

In contrast to the family of deoxyribonucleoside kinases characterized from several species, *Drosophila melanogaster* contains a single highly efficient deoxyribonucleoside kinase (*Dm*-dNK) that is able to phosphorylate all four natural deoxyribonucleosides as well as several clinically important antiviral and anticancer nucleoside analogs (Munch-Petersen et al., 1998; Johansson et al., 1999). We recently evaluated *Dm*-dNK as a suicide gene by expressing the enzyme in human cancer cell lines using a replication-deficient retroviral vector (Zheng et al., 2000). We have shown that *Dm*-dNK can be expressed in human cells and that the enzyme retains its enzymatic activity. The cells expressing *Dm*-dNK exhibited an increased sensitivity to several cytotoxic nucleoside analogs, among which (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) was one of the most efficient prodrug candidates.

A major limitation of gene therapy for cancer at present is the inability to transduce all the cancer cells in vivo (Moolten, 1986; Moolten and Wells, 1990; Culver et al., 1992; Freeman et al., 1993; Vile et al., 1994; Smythe et al., 1995; Roth and Cristiano, 1997). Bystander killing is thus critical for the eradication of tumors (Roth and Cristiano,

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ABBREVIATIONS: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; *Dm*-dNK, *Drosophila melanogaster* deoxyribonucleoside kinase; HSV-1 TK, herpes simplex virus type-1 thymidine kinase; GCV, ganciclovir; TBS, Tris-buffered saline.

1997). However, suicide genes are also used in clinical protocols of allogenic bone marrow transplantation (Link et al., 2000). The HSV-1 TK gene can be transfected ex vivo into donor T lymphocytes before their infusion into patients. GCV can subsequently be administered to destroy the allogenic T lymphocytes if graft-versus-host disease occurs. In this case, bystander effect is probably unimportant and should even be avoided.

In the present study, we investigated the bystander effect of BVDU in a *Dm*-dNK-transduced thymidine kinase-deficient osteosarcoma cell line and the MIA PaCa-2 human pancreatic adenocarcinoma cell line. We also studied the ability of hydroxyurea, a ribonucleotide reductase inhibitor, to enhance the cytotoxicity and the efficiency of bystander killing of *Dm*-dNK-transduced cells treated with BVDU.

Materials and Methods

Cell Culture and Retroviral Transduction. TK-deficient osteosarcoma cells was a kind gift from Professor J. Balzarini (Rega Institute, Leuven, Belgium). MIA PaCa-2 human pancreatic adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were cultured at 37°C in a humidified incubator with a gas phase of 5% CO_2. The production of recombinant replication-deficient retroviral vectors has been described in detail previously (Zheng et al., 2000). The cells were transduced with the retrovirus-containing medium mixed with 4 μg/ml polybrene. The cells were incubated for 48 h and then cultured continuously for 3 weeks in the presence of 1.0 mg/ml Geneticin (Invitrogen).

Generation of Mouse Polyclonal Antibodies and Western Blot Analysis. *Dm*-dNK was expressed in the BL21 *Escherichia coli* strain with an N-terminal polyhistidine tag and was purified by TALON resin affinity chromatography (CLONTECH, Palo Alto, CA). Then, 2.0 μg of fusion protein in 300 μl of phosphate-buffered saline was subcutaneously injected into three 4-week-old female BALB/c mice with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). This was followed by a booster injection 10 days later of the same amount of fusion protein in Freund's incomplete adjuvant (Sigma), which was injected in the same manner. Two weeks after the booster injection, 3 ml of blood was retrieved and allowed to clot. The serum was collected and stored at −20°C.

Protein extracts were prepared as described previously (Soderlund and Arner, 1994). The protein concentration of cell extracts was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The protein extracts were separated by 1.2% SDS-polyacrylamide gel electrophoresis and electrotransfered to the nitrocellulose membrane at 35 V overnight. The membranes were blocked for 1 h at room temperature with 1% bovine serum albumin in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The membranes were incubated for 1 h at room temperature with the affinity-purified *Dm*-dNK antibody and washed three times with TBS. A secondary alkaline phosphatase-conjugated anti-mouse IgG antibody diluted in a ratio of 1:5000 (Sigma) was applied for 1 h, after which the membranes were again washed in TBS. The alkaline phosphatase immobilized on the membrane was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

Cell Proliferation Assays. (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) was a gift from Professor J. Balzarini. The cells were plated at ≈2000 cells/well in 96-well plates. BVDU with and without hydroxyurea (120 μM) was added after 24 h, and the medium containing the BVDU (with and without hydroxyurea) was changed once during the experiment. Cell survival was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Molecular Biochemicals, Summerville, NJ) after 2 to 3 days of drug exposure. Each experiment was performed in triplicate. The IC_{50} value of the investigated compounds was calculated as the mean value of the experiments.

Bystander Effect. The protocol used for the bystander experiments is similar to protocols described previously (Denning and Pitts, 1997; Qiao et al., 2000). Tumor cells expressing *Dm*-dNK were mixed at different ratios with their respective parental cell lines. To promote cell contacts, the mixed cells were plated in 24-well plates at 3×10^5 cells/well. The following day, confluent cells were treated with BVDU ranging from 0.001 to 100 μM. After another 24-h incubation, cells were trypsinized and a 1:100 dilution of the cells was distributed into 96-well plates in five replicates. Cells were cultured subsequently in the presence of BVDU for 2 to 3 days until cells without BVDU reached confluency. The proliferation of the cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. We calculated the inhibition of bystander cells proliferation using a method described previously (Qiao et al., 2000). In the cultures containing *A*% dNK-expressing cells, the relative cell proliferation of *Dm*-dNK-expressing cells (*Dm*-dNK cell proliferation × *A*) and untransfected cells [(untransfected cell proliferation × (100 − *A*)] was subtracted from the relative proliferation of the cultures containing mixtures of *Dm*-dNK-expressing cells and untransfected cells [cell mixture proliferation − *A* × (*Dm*-dNK cell proliferation) − (100 − A) × (untransfected cell proliferation)]. The inhibition of bystander cell proliferation was expressed as a percentage relative to cells cultured without BVDU; i.e., −50% reflects a 50% decrease in proliferation of bystander cells compared with the inhibition of proliferation detected in the cells in which no bystander effect occurred.

Results

Expression of *Dm*-dNK in Cancer Cells. A thymidine kinase-1 (TK1)-deficient human osteosarcoma cell line and a MIA PaCa-2 human pancreatic adenocarcinoma cell line were transduced with replication-deficient recombinant retrovirus with and without the *Dm*-dNK cDNA. A polyclonal population of stably transduced cells was obtained by Geneticin selection. Western blot analysis with mouse polyclonal anti-*Dm*-dNK antibodies detected a band of ~28 kDa in the cells transduced with *Dm*-dNK, but not in the cells transduced with the control vector (Fig. 1A). The specificity of the antibodies was verified using a Western blot analysis on recombinant *Dm*-dNK and the sequence-related human deoxyribonucleoside kinases deoxycytidine kinase, deoxyguanosine kinase, and thymidine kinase-2 (Fig. 1B). The anti-*Dm*-dNK antibodies detected the *Dm*-dNK protein and did not cross-react with the human nucleoside kinases. To verify that the *Dm*-dNK retained its enzymatic activity when expressed in human cells, we determined the activity of thymidine phosphorylation in crude cell protein extracts. Compared with their parent untransduced cell line, the human osteosarcoma cells and the pancreatic adenocarcinoma cells expressing *Dm*-dNK showed ≈100-fold and ≈30-fold increases in thymidine kinase activity, respectively (Zheng et al., 2000).

Bystander Effect of BVDU in *Dm*-dNK Expressing Cells. Cells expressing *Dm*-dNK (*Dm*-dNK$^+$) were mixed at indicated ratios with cells from their respective parental cell

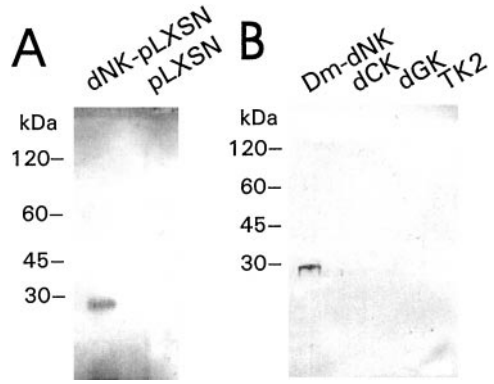

Fig. 1. Western blot analysis of *Dm*-dNK expression. A, Western blot analysis protein extracts from osteosarcoma cells transduced with the pLXSN vector or the vector expressing *Dm*-dNK. B, the mouse polyclonal antibodies reacted with recombinant *Dm*-dNK and no cross-reactivity with the human nucleoside kinases deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), and thymidine kinase-2 (TK2).

line (*Dm*-dNK$^-$). To promote cell contacts, the mixed cells were initially plated in 24-well plates. The next day, BVDU was added to the confluent cells at concentrations ranging from 0.001 to 100 μM. After another 24-h incubation, the cells were trypsinized and a dilution of the cells was distributed into 96-well plates; exposure to BVDU was continued for another 2 to 3 days. A bystander effect was found in the osteosarcoma cell line (Fig. 2A), but not in the MIA PaCa-2 cells (Fig. 2B). At BVDU concentrations of 1 μM or higher, a bystander effect was seen at all investigated different mixtures of *Dm*-dNK$^+$ and *Dm*-dNK$^-$ osteosarcoma cells, whereas no bystander effect was seen at lower concentrations of BVDU.

Effects of Hydroxyurea on the Cytotoxicity of BVDU. The ribonucleotide reductase inhibitor hydroxyurea has been shown to enhance cell killing and bystander effects in cells transduced with HSV-1 TK and treated with GCV (Boucher et al., 2000). To determine whether hydroxyurea had an effect on the BVDU-mediated cell death, we measured the sensitivity of cells expressing *Dm*-dNK in both TK1-deficient osteosarcoma and wild-type MIA PaCa-2 human pancreatic adenocarcinoma cell lines. Hydroxyurea alone was not toxic to the cells up to concentrations of 150 μM (Fig. 3), and we therefore decided to use 120 μM hydroxyurea in the combination experiments. The IC_{50} value was ≈150-fold lower for the *Dm*-dNK-expressing osteosarcoma cells incubated with BVDU in the presence of 120 μM hydroxyurea than for cells incubated with BVDU alone (Table 1). In the MIA PaCa-2 cell line, the IC_{50} value for BVDU was 50-fold lower in the presence of hydroxyurea.

Effect of Hydroxyurea on the Bystander Effect of BVDU. Mixtures of *Dm*-dNK$^-$- and *Dm*-dNK$^+$-transduced cells, with and without 120 μM hydroxyurea, were cultured with different concentrations of BVDU to study the bystander effect. The osteosarcoma cells showed a bystander effect that gradually increased with higher doses of BVDU. The bystander effect was more pronounced in the presence of hydroxyurea, with a highly significant difference compared with the bystander effect of BVDU in cell cultures without hydroxyurea (Table 2). We found that 10% *Dm*-dNK-expressing cells induced cell death in the presence of hydroxyurea to a degree similar to that obtained obtained with 50% *Dm*-dNK-expressing cells without hydroxyurea. For the MIA PaCa-2 cell line, there was no bystander effect in either the absence or the presence of hydroxyurea (data not shown).

Discussion

In this study, we investigated the cytotoxicity and the bystander effect of a novel suicide gene/prodrug combination: *Dm*-dNK and BVDU. Our data demonstrate a BVDU-mediated bystander cell killing in *Dm*-dNK-transduced osteosarcoma cells that could be enhanced by subtoxic concentrations of hydroxyurea. In a *Dm*-dNK-transduced human pancreatic adenocarcinoma cell line, MIA PaCa-2, we showed that hydroxyurea increased the cytotoxicity of BVDU, but a bystander effect was not detected in these cells. In a previous study, we demonstrated that BVDU was an efficient substrate for both HSV-1 TK and *Dm*-dNK (Johansson et al., 1999), and the compound has previously been investigated as a prodrug in HSV-1 TK-transduced cells. Although BVDU did not show any bystander effect in HSV-1 TK-transduced osteosarcoma cells (Degreve et al., 1999), we detected a clear bystander effect in the *Dm*-dNK-transduced osteosarcoma cells in the present study. The high catalytic rate of *Dm*-dNK is one possible mechanism to explain the differences in bystander effects for BVDU when activated by different enzymes in the same cell line. However, the bystander effect of BVDU in our study is not as pronounced as the bystander effect of GCV in HSV-1 TK-transduced cells, and GCV remains unique among the nucleoside analog prodrugs regarding its high efficiency in bystander cell killing. No bystander killing was observed for BVDU in the MIA PaCa-2 pancreatic adenocarcinoma cell line transduced with *Dm*-dNK. This cell line has pre-

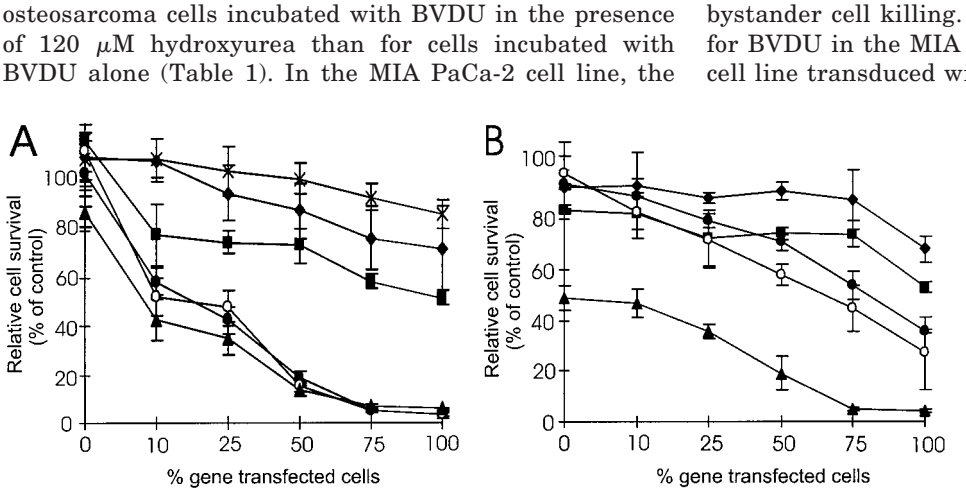

Fig. 2. In vitro bystander effect. A, osteosarcoma cells. B, MIA PaCa-2 pancreatic adenocarcinoma cells. Nontransduced and *Dm*-dNK gene-transduced cells were mixed in various percentages (0, 10, 25, 50, 75, and 100% *Dm*-dNK gene-transduced cells), incubated in the presence of the BVDU at 0.001 (except MIA PaCa-2) (×), 0.01 (♦), 0.1 (■), 1 (●), 10 (□), and 100 μM (▲). Data are expressed as a proliferation percentage relative to the proliferation of cells in the absence of BVDU and are the average of five values ± S.D.

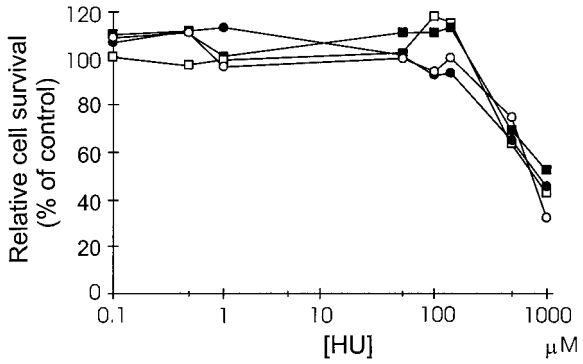

Fig. 3. Hydroxyurea sensitivity of TK1-deficient osteosarcoma cells (■), osteosarcoma cells transduced with *Dm*-dNK (□), MIA PaCa-2 pancreatic adenocarcinoma cells (●), and MIA PaCa-2 cells transduced with *Dm*-dNK (○).

viously been shown to exhibit poor bystander killing when using HSV-1 TK/GCV. The MIA PaCa-2 cell line has also shown very low levels of expression of mRNA for both connexin-43 and connexin-26, which are the main proteins of gap junctions (Yang et al., 1998).

Several mechanisms may be responsible for bystander effects in vivo, and at least two mechanisms have been shown to mediate bystander killing in HSV-1 TK-transduced cells in vitro (Aghi et al., 2000). These include the transfer of phosphorylated GCV through intercellular gap junctions and the phagocytosis of apoptotic vesicles containing GCV metabolites from dying HSV-1 TK-transduced cells (Bi et al., 1993; Freeman et al., 1993; Seachrist, 1994; Fick et al., 1995). The importance of gap junctions is supported by studies demonstrating that the bystander effect correlates with the extent of gap junctions between cells (Fick et al., 1995) and the fact that neuroblastoma and pheochromocytoma cells that lack endogenous junctional conductance show no bystander effect unless they are transfected with connexin genes (Vrionis et al., 1997).

TABLE 1
Sensitivity (IC_{50}) of two cell lines to BVDU with and without hydroxyurea

	IC_{50}		
	Untransduced cells	Dm-dNK	Dm-dNK + Hydroxyurea
		μM	
Osteosarcoma	>10	0.028	0.00018
MIA PaCa-2	>10	0.12	0.0027

Also, drugs that up-regulate gap junctions, such as retinoic acid (Park et al., 1997), augment the HSV-1 TK/GCV bystander effect. Dieldrin, which decreases gap junctions, reduces this effect (Touraine et al., 1998). The role of gap junctions for the in vivo bystander effect has been verified through the transfection of rat glioma cells with connexin-43 cDNA. The experimental tumors could be completely eliminated when only 25% of the cells expressed HSV-1 TK, whereas tumor cells expressing low endogenous levels of connexin-43 could not be eliminated even when 50% of the cells expressed HSV-1 TK (Dilber et al., 1997).

Hydroxyurea increases the GCV sensitivity of cells expressing HSV-1 TK (Boucher et al., 2000). Hydroxyurea reduces the intracellular dGTP level, and the enhancement of GCV-mediated toxicity was suggested to be the result of an increased GCV-TP/dGTP ratio. We found a marked increase in cytotoxicity of BVDU, similar to that of GCV, when the compound was administered in combination with subtoxic concentrations of hydroxyurea. Although the total dTTP pool increases in cells incubated with hydroxyurea (Collins and Oates, 1987), the enhanced sensitivity to pyrimidine analogs is probably a result of a decrease in the de novo dTTP synthesis, which could favor the salvage of deoxyribonucleosides and nucleoside analogs. It is presently not known how the expression of *Dm*-dNK influences the dNTP pools in cells. This enzyme is unique in its efficient phosphorylation of all four natural deoxyribonucleosides, and future studies should reveal how such a highly active enzyme affects the dNTP pools and the supply of DNA precursors.

Dm-dNK has a broad substrate specificity, and several cytotoxic compounds have been identified as substrates for the enzyme. Our study suggests that the *Dm*-dNK/BVDU combination should be further evaluated in applications in which a bystander effect is not desired (i.e., in allogenic bone marrow transplantation and in other cell therapy protocols). For these applications, the inability of *Dm*-dNK to activate GCV or acyclovir should be an advantage because these patients often have herpes virus infections. For the treatment of solid tumors, other substrates for *Dm*-dNK should be evaluated that may have more efficient bystander cell killing. It is important to realize that studies of bystander effects performed in vitro may be of limited value to predict the in vivo situation. However, they are an important step toward a better understanding of

TABLE 2
Inhibition of bystander cell proliferation
The numbers shown are the calculated relative inhibition of bystander cell proliferation (average ± S.D.) compared with control cells as described under *Materials and Methods*. ost^-/ost^+, ratio of untransfected osteosarcoma cells (ost^-) and Dm-dNK expressing osteosarcoma cells (ost^+).

	BVDU				
	10 μM	1 μM	0.1 μM	0.01 μM	0.001 μM
			%		
ost^-/ost^+(90/10)	−49 ± 3	−35 ± 11	−33 ± 13	2 ± 4	3 ± 6
ost^-/ost^+(90/10) + HU	−46 ± 9	−60 ± 13	−44 ± 12	−36 ± 7	−21 ± 4
ost^-/ost^+(75/25)	−38 ± 9	−36 ± 10	−26 ± 5	−6 ± 4	1 ± 4
ost^-/ost^+(75/25) + HU	−46 ± 5	−44 ± 16	−41 ± 19	−35 ± 7	−21 ± 11
ost^-/ost^+(50/50)	−43 ± 5	−35 ± 6	−11 ± 7	−3 ± 9	3 ± 7
ost^-/ost^+(50/50) + HU	−38 ± 1	−34 ± 9	−34 ± 9	−33 ± 8	3 ± 6
ost^-/ost^+(25/75)	−26 ± 2	−23 ± 3	−9 ± 3	−5 ± 4	1 ± 6
ost^-/ost^+(25/75) + HU	−21 ± 1	−22 ± 7	−19 ± 2	−34 ± 14	−19 ± 12

HU, hydroxyurea.

the basic mechanisms of prodrug activation by suicide genes and for the development of novel therapeutic suicide gene/prodrug combinations.

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