

Targeted therapy of CEA-producing cells by combination of *E. coli* cd/HSV1-tk fusion gene and radiation

Research Article

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Summary

To enhance the specific cytotoxic effects caused by the transfer of the *E. coli* cytosine deaminase (cd) and HSV1-tk to CEA (carcinoembryonic antigen)-producing cells, the expression of the cd-tk fusion gene, driven by the CEA promoter, was investigated followed by treatment with 5-FC and GCV in combination with radiation. The expression vector pCEAc-d-tk, based on pcDNA3, was introduced into CEA-producing cells using liposomes. In CEA-producing cells, the CEA promoter could efficiently drive the expression of the fusion suicide gene. The expression activity of the *E. coli* cd gene driven by the CEA promoter was about three times higher than that driven by the CMV promoter in transfected LoVo cells. A combination of 5-FC and GCV could cause higher cytotoxicity to the cells expressing CD and TK than the use of a single prodrug alone. The cytotoxic effect after combining the two prodrugs with radiation was the highest among all treatments *in vitro*. *In vivo*, the result of a subrenal capsule assay showed that the inhibition rates for 5-FC (0.5 mg/g) and GCV (0.1 mg/g) to GLC-82 cells transfected with pCEAc-d-tk were 18.04% and 55.00%, respectively. A combination of the prodrugs at the same dose resulted in a 152.50% inhibition rate. In addition, the bystander effect exerted by the pCEAc-d-tk/5-FC+GCV system *in vitro* was greater than that induced by cd/5-FC or tk/GCV alone.

I. Introduction

CEA (carcinoembryonic antigen)-positive tumors are common clinically. At present, there are no efficient therapeutic measures, especially for the patients who are in the mid- or final stages of this disease. Gene therapy may show its strength as an effective method for treating this carcinoma. The herpes simplex virus type I thymidine kinase (HSV1-tk) and the *Escherichia coli* cytosine deaminase (*E. coli* cd) genes are commonly used as suicide genes. The expression products of these two non-mammalian genes are two enzymes, HSV1-TK and *E. coli* CD, which can convert the nontoxic prodrugs, ganciclovir

(GCV) and 5-fluorocytosine (5-FC), respectively, into metabolites highly toxic to the genetically-modified tumor cells. Experimental results showed that use of the *E. coli* cd/5-FC or of the HSV1-tk/GCV systems could inhibit the growth of CEA-producing tumor cells *in vitro* and *in vivo* (DiMaio et al., 1994; Richards et al., 1995; Lan et al., 1997). However, it has been observed that some tumor cells were resistant to *E. coli* cd/5-FC or HSV1-tk/GCV (Golumbek et al., 1992; Mullen et al., 1994; Bennedetti et al., 1997).

The treatment efficiency of the suicide gene/prodrug system mostly depends on the expression efficiency of the

introduced suicide gene in the tumor cells. Therefore, the promoter used to drive the expression of a suicide gene is very important. The most commonly used promoters are viral promoters. However, viral promoters are easily inactivated in mammalian cells, resulting in an unstable and low-efficiency expression of a suicide gene. In addition, viral promoters lack the cell-specific activities, which could repress expression of a suicide gene in normal cells. Using a retrovirus vector, high expression of the introduced gene was found only in a small subset of the transfected cells; most of the transfected cells did not display the expression product of the introduced gene (Mullen, 1994).

Over-expression of the CEA gene was a special feature for CEA-positive tumors, and the high level of CEA in physiological fluids has been used for early diagnosis and as a marker of treatment efficiency (Shively & Beatty, 1985; Thomas et al., 1990). Although there was some CEA expression in the normal epithelial cells of the colon, the level was very low (Baranov et al., 1994; Egan et al., 1977). The over-expression of CEA gene resulted from the activated CEA promoter and not from a mutation in the CEA promoter causing its upregulation (Schrewe et al., 1990; Jothy et al., 1993). The CEA promoter occupies a stretch of 420-bp upstream of the translation start site of the CEA gene (Chen et al., 1995; Richards et al., 1995). In CEA-positive cells, specific trans-acting elements are present which activate the CEA promoter. Because of these properties, the CEA promoter could be used to drive the expression of therapeutic genes only in CEA-positive tumor cells; in this context, the *E. coli* *cd* and HSV1-*tk* genes have been used (Osaki et al., 1994; Richards et al., 1995).

The mechanism of cell killing by radiation proceeds via damage of the strands of cellular DNA. Cells able to repair the damaged DNA will survive. Because the *E. coli* *cd/5-FC* and HSV1-*tk/GCV* systems kill cells through inhibition of DNA synthesis, they could also be used as radiosensitizing agents. It was found that both the *E. coli* *cd/5-FC* and HSV1-*tk/GCV* systems could enhance the sensitivity of cells to radiation (Khil et al., 1996; Rogulski et al., 1997).

Here we investigate whether a combination of *E. coli* *cd/5-FC*, HSV1-*tk/GCV* and radiation exert a greater cytotoxic effect to tumor cells, especially to CEA-producing tumor cells. Use of the CEA promoter can limit the expression of the fusion suicide gene in CEA-producing cells. Under these circumstances, treatment with the two prodrugs and application of a low-dose radiation had a much higher cytotoxicity to the tumor cells while minimizing side-effect to normal cells.

II. Results

A. Enzymatic activities of CD and TK

Using PCR, a single fragment of about 450-bp was observed on 2% agarose gels (Figure 1A). The same fragment could be amplified from different healthy donors. Sequencing analysis showed that there was only one base mismatch in the CEA promoter fragment of pCEA (Figure 1B), compared with the CEA promoter sequence published by others (Richards, et al., 1993).

CEA quantitation in the cell lines was measured by RIA (radioimmuno assay). CEA concentrations were found to be different in different cell lines (Table 1): LoVo and HT-29 cells displayed the highest levels of CEA (581.4 and 316 nm/mg of cellular lysate, respectively). On the contrary, CEA was not detected in BEL-7402 cells. When *cd^c* (*E. coli* complementary *cd* gene obtained by PCR, but using the antisense primer but leaving the native stop codon unchanged) was used as an indicator enzyme, the activity of CEA promoter driving its expression was 3.14 times higher than that of CMV promoter in LoVo cells (Table 2).

Through sequence analysis, our *cd* sequence was the same as the *E. coli* *cd* sequence of the GenBank No s56903 except that of the start and the stop codons which had been changed on purpose. Compared to the HSV1-*tk* of Genbank No. v00470, one base mismatch leads to the change of the 17th alanine to valine in the *tk* used in our experiment (data not shown).

In transfected BEL-7402 cells, no CD activity was detected. In pCEA*cd*-*tk* transfected cells, the activities of CD and TK were measured respectively (Table 3). The results indicate that all CEA-producing cells have higher enzymatic activities than the corresponding parental cells. In non-transfected BEL-7402 cells, there is a low relative activity of TK. It is the activity of cellular TK, not HSV1-TK, because ³HdT was used as the substrate.

Table 1. Concentration of CEA protein in the tumor cell lines

Cell line	Concentration of CEA (ng/mg of cellular lysate)
LoVo	581.4
HT-29	316.8
SGC-7901	60.6
GLC-82	80.2
BEL-7402	BT*

The quantity of CEA protein was measured by use of RIA (Radioimmuno assay) method. * below the threshold of 5 ng.

Figure 1. A. Amplification of CEA promoter. The PCR products were separated on 2% agarose gel. Lane 1, DNA molecular weight marker, Lambda DNA/EcoRI+HindIII; lane 2-9, PCR products from peripheral blood cell genomic DNA of healthy individuals. **B.** Comparison of the CEA promoter sequence with published CEA DNA sequence in the 5' non-translation region. Query: the sequence of CEA promoter used in the experiment; subject: part of the published CEA DNA sequence (Genbank No: z21818).

Table 2. Enzymatic activities of CD in tumor cells (specific activity)

Cell line	Parental	Transfected with pCEAcde	Transfected with pcDNA3cdc
LoVo	0	2748	875
HT-29	0	2034	-
SGC-7901	0	670	-
GLC-82	0	714	-
BEL-7402	0	0	-

Specific activity was defined as nmol of cytosine deaminated/min/mg protein. It was measured spectrophotometrically as a decrease in absorbance at 285 nm, in a 1-ml assay mixture containing cell extract in 50 mM Tris-HCl, pH 7.3, 0.5 mM cytosine. The product was estimated using a molar extinction coefficient 1.038×10^4 litre/mol/cm.

Table 3. Enzymatic activities of CD and TK in the cells transfected with pCEAcde-tk

Cell line	Enzymatic activity	
	CD*	TK#
LoVo	2516	238.1
HT-29	1603	149.2
SGC-7901	421	84.7
GLC-82	507	69.4
BEL-7402	0	10.8

*: specific activity; #: relative activity to TK activity in the pcDNA3tk-transfected cells.

Figure 2. Additive cytotoxic effect of combined use of 5-FC and GCV to tumor cells expressing cd-tk. SEMs (standard error means) were presented by error bars(n=3).

Figure 3. In vitro bystander killing effect of pCEAc-dtk/5-FC+GCV. The pCEAc-dtk transfected LoVo cells were mixed with non-transfected LoVo cell in different portions. The mixed cells were seeded on a 96-well plate at a density of 2×10^3 cells per well. 24 hrs later, the cells were exposed to 0.5 μ M GCV and 100 μ M 5-FC. After the cells were incubated for 72 hrs, the surviving rates were measured by MTT assay. Bars, SEMs (n=3).

B. Cytotoxicity of 5-FC and GCV to tumor cells expressing CD and TK

CEA-producing cells transfected with pCEAc-dtk become more sensitive to 5-FC and GCV than parental cells as deduced from growth inhibition *in vitro* measuring the IC_{50} (concentration of 50 % growth inhibition) (**Table 4**). Use of 5-FC in combination with GCV has a remarkable additive cytotoxic effect to CEA-producing cells expressing CD and TK (**Figure 2**). In addition, the *in vitro* pCEAc-dtk/5-FC+GCV system has a higher bystander effect than cd/5-FC or tk/GCV (**Figure 3**). In the 20% group, treatment with 5-FC plus GCV produces 5.4%

surviving rate. When the same dose of 5-FC or GCV was used alone, the survival rates were 40.2% and 56.7%, respectively. The subrenal capsule assay (SRCA) result indicates that the tumor inhibition rate is much higher when using a combination of the two prodrugs in nude mice (**Table 5**).

C. Radiosensitization of pCEAc-dtk/5-FC+GCV

When 100 μ Mol 5-FC or 0.5 μ Mol GCV is added to LoVo cells transfected with pCEAc-dtk, 6.5 Gy and 4.9

Table 4. The IC50 of tumor cells to 5-FC and GCV

Cell line	IC50±SD(μ M)		Ratio IC50#	
	5-FC	GCV	5-FC	GCV
LoVo*	67.2± 26.8	0.75± 0.16		
LoVo	9650.34± 563.00	25250.60±430.85	143.61	3360.80
HT-29*	162.70± 56.20	0.86± 0.24		
HT-29	9580.50± 762.58	840.70±125.42	58.88	977.56
SGC-7901*	287.57± 40.48	17.30± 5.16		
SGC-7901	10865.20± 481.82	890.91±231.73	37.78	51.50
GLC-82*	232.10± 81.34	12.83± 7.51		
GLC-82	13720.63±2407.38	950.60±124.30	59.12	74.09
BEL-7402*	9080.85± 375.31	678.59± 35.70		
BEL-7402	11070.14±2512.17	780.41±147.20	1.22	1.15

Cells were seeded at a density of 2×10^3 cells/well on 96-well plates. Different concentrations of 5-FC, GCV were added. After 72hrs, the percentage of growth inhibition was measured by the MTT assay. The results represent mean±SD(n=3).

IC50=the concentration of 50% growth inhibitory rate.

* cells transfected with pCEAc-dtk;

parental cell IC50/transfected cell IC50 to 5-FC or GCV.

Table 5. *In vivo* growth inhibition of pCEAc-dtk transfected GLC-82 cells by 5-FC and GCV

Drug	Dose (mg/gm)	Schedule	Do	Dn	Dn-Do	Inhibition rate(%)
Control	0.04*	1/d×2	40.5±6.26	43.8±10.36	3.3± 9.01	----
5-FC	0.5	1/d×2	39.7±5.93	42.5±12.68	2.7±11.34	18.04
GCV	0.1	1/d×2	36.6±5.55	38.1±11.3	11.5± 9.44	55.00
5-FC+GCV	0.5+0.1	1/d×2	41.9±4.68	40.2±16.6	-1.7±16.91	152.50

The prodrugs were given by the intraperitoneal injection.

*: ml of 0.9% NaCl per gram;

Do: tumor volume before transplantation, Dn: tumor volume after the animal was sacrificed.

Gy, respectively, were required to reduce the surviving fraction to 0.01 (**Figure 4A**). When a combination of the same dose of 5-FC plus GCV was used, only 4.2 Gy were required to obtain the same survival fraction. The pCEAc-dtk/5-FC+GCV system had a similar effect on GLC-82 cells (**Figure 4B**).

III. Discussion

It is possible that the mutation in the CEA promoter can affect its activity and cell-specificity. In our experiments, the CEA promoter is obtained using a high fidelity DNA polymerase. The CEA promoter shows a higher activity in CEA-producing cells. In LoVo cells its activity was 3.14 times higher than that of the CMV promoter (using the *E. coli* CD as the indicator). The sequence of the CEA promoter used in our experiments is almost identical to that of the CEA promoter sequence published before (Richards, et al., 1993) except one base difference, 109 A→G (**Figure 1B**). It was found that the

essential part of CEA promoter was located between nucleotides 295-318 (Richards, et al, 1995). Through footprinting, Chen, et al (1995) and Hauck and Stanners (1995) found that there were 5 FP (footprinting) regions in the cis-acting sequence of the CEA promoter, in which FP1-4 represented the positive regulatory elements whereas FP5 (-568 to -560, where +1 is the start of translation) represented the negative regulatory elements. Sp1 and Sp1-like factors could bind to Fp1, FP2 and FP3. The protein bound to FP4 was AP4. The only different base in the CEA promoter used in our experiments is in the FP4 region. Although the FP4 region was not an essential part of the CEA promoter, it may affect the activity of the CEA promoter. Using a common Taq DNA polymerase, an active CEA promoter could be obtained (DiMaio et al., 1994), but a low activity CEA promoter was observed (Osaki et al., 1994). It is not clear which bases play a key role in the CEA promoter activity. It is possible that a more efficient CEA promoter can be constructed by changing some bases in the CEA promoter

sequence, which may be much better suited for targeting expression of a suicide gene to CEA-producing tumor cells.

Although the essential sequence for an active CEA promoter is known (Richards et al., 1995), the mechanism of activating CEA promoter is unclear. Our results indicate that the activities of CD and TK in different CEA-producing cell lines transfected with pCEAc-d-*tk* are different. The enzymatic activity shows a positive relationship to the concentration of CEA in the cells. An active CEA promoter is determined by the interaction of a cis-acting sequence with trans-acting elements. We found that the nuclear proteins binding to the CEA promoter were different between LoVo and BEL-7402 using gel mobility shift assays (data not shown). The different enzymatic activities may reflect the different interactions involving these elements.

Combined therapy to tumors can enhance the cytotoxicity and beneficial effect from each therapeutic regime. Using cotransfection of cells with HSV-*tk* and *E. coli* *cd*, Uckert, et al. (1998) found that the combination of the two genes was the most effective for killing tumor cells both *in vitro* and *in vivo*, and only this combination could cause complete eradication of tumors *in vivo*. Rogulski et al. (1997) revealed that the combined use of *cd*-*tk*/5-FC+GCV and radiation had a strong cytotoxic effect to 9L tumor cells. The best way to treat tumors is to kill only tumor cells without any severe damage to healthy cells. So it is important to limit the expression of a suicide gene only in tumor cells before using the prodrug. At present, two ways, targeting vectors and targeting transcription (see review by Miller & Whelan, 1997), can be used. We used the strategy of targeting transcription, and the pCEAc-d-*tk*/5-FC+GCV system showed a strong cytotoxic effect to the CEA-producing cells. In addition, high concentration of GCV or 5-FC could cause remarkable nonspecific toxicity to nontransfected cells (Beck, et al., 1995, Cool, et al., 1996). Use of the combination of these two systems will reduce the dose of each prodrug, whereas the cytotoxic effect can be enhanced. In our experiments the doses of 5-FC and GCV are much lower than the "safe" concentrations of these in human blood. If higher doses of the prodrugs are used, the pCEAc-d-*tk*/5-FC+GCV might kill tumor cells even more efficiently reducing the possibility of converting tumor cells to become resistant.

The mechanisms of the bystander effect of *E. coli* *cd*/5-FC and HSV1-*tk*/GCV are not completely clear, but clear differences between these two systems have been observed (Denning & Pitts, 1997). The combined use of the two systems could promote the bystander effect (Rogulski, et al., 1997, Uckert, et al., 1998). In agreement

with this we found that the bystander effect of the combined use is enhanced *in vivo*.

Although HSV1-*tk*/GCV, *E. coli* *cd*/5-FC system could effectively kill tumor cells *in vitro* and *in vivo*, the efficiency between these two systems were different to some kinds of tumors. *E. coli* *cd*/5-FC therapy was more effective than HSV1-*tk*/GCV to pulmonary adenocarcinoma (Hoganson et al., 1996). *In vivo*, human colorectal carcinoma cells were more effectively eradicated by *E. coli* *cd*/GCV than HSV1-*tk*/GCV (Trinh et al., 1995). Most gastric-intestinal and lung carcinomas are CEA-positive. On the other hand, tumor microenvironment can determine the cell radiosensitivity, but the sensitivity of tumor cells to radiation also is dependent on intrinsic cellular factors. Both HSV1-*tk*/GCV and *E. coli* *cd*/5-FC could alter the cellular factors, and enhance the radiosensitivity (Kim et al., 1994, 1995; Khil et al., 1996; Rogulski et al., 1997). Most CEA-positive tumor cells, for example pulmonary adenocarcinoma cells, are not sensitive to radiation. Therefore, it is much more effective to use a combination of these two systems to kill these tumor cells.

There were some limitations for treating pulmonary adenocarcinoma cells by use retrovirus-mediated HSV-*tk* gene transfer (Zhang et al, 1997). Song et al. (1997) found that injection of a pcDNA3-liposome mixture could cause the highest expression of an exogenous gene in mouse lungs. In addition, the most common reason for mortality of patients with colon carcinoma is hepatic metastases. In normal lung and liver tissues, the CEA gene is not expressed. If pCEAc-d-*tk* is non-specifically transfected into these normal cells, the suicide gene will not be expressed since the CEA promoter is in an inactive state. After use of prodrug, no toxic metabolite of the prodrug will be produced in the normal cells, thus reducing the side effects of suicide gene/prodrug therapy to normal cells. The therapeutic system, pCEAc-d-*tk*/5-FC+GCV accompanied with low dose of radiation, may become a useful tool for the eradication of CEA-producing tumors.

IV. Materials and methods

A. Vector construction

Two primers were used to amplify the CEA promoter from the genomic DNA of peripheral blood cells from healthy blood donors, 5'-GTA TCG CGA ATC ATC CCA CCT TCC CAG AG-3' (sense), 5'-GGG AAG CTT TGT CTG CTC TGT CCT CCT C-3' (antisense). The high-fidelity Pwo polymerase (Boehringer Mannheim Co.) was used to amplify a 438-bp CEA promoter. The amplified fragment was cut with *Nru*I and *Hind*III, and then the CMV promoter in pcDNA3 (Invitrogen) was replaced with this fragment, resulting in the vector pCEA, in which the CEA promoter fragment was ensured by direct

Figure 4. The radiative enhancing effect of pCEAc-dtk/5-FC+GCV. A: LoVo; B: GLC-82. 2×10^3 cells/well were seeded on 96-well plates, and then $100 \mu\text{M}$ 5-FC and $0.5 \mu\text{M}$ GCV were added. 72hrs later, the cells were irradiated with different doses of X-rays. After 6 days, the cell number in each well was estimated by MTT assay according to the standard calibration curves. SEMs (n=3) were omitted for clarity.

dideoxynucleotide sequencing. The primers, (sense) 5'-GGG AAG CTT ACC ATG TCG AAT AAC GCTTTA C-3' (with a HindIII cut site in 5' end) and (antisense) 5'-CGC GGATCC TCC ACG TTT GTA ATC GAT GGC-3'(with a BamHI cut site in 5' end) were used to amplify the *E. coli* cd gene from chromosomal DNA of JM109 bacteria. In the sense primer, the initial context was changed into the Kozak sequence (Kozak, 1986). The stop codon (TGA) of *E. coli* cd was changed into GGA (encoding for glycine), leading to read through downstream HSV1-tk gene. The other two primers were used to amplify the HSV1-tk gene from the plasmid pHSV106 (GIBCO-BRL). The sense primer was 5'-CGC GGA TCC GGC GGG GGC GGT GGA GGA GGG GGT ATG GCT TCG TAC-3', in which there was a BamHI cut site and eight codons for glycine. The antisense primer was 5'-CGG GAA TTC CCT TCC GGT ATT GTC TCC TTC CGT-3'(with EcoRI cut site) (Rogulski et al., 1997). The ligation and identification of inserted fragments by using restriction enzyme analysis was carried out according to methods described (Sambrook et al., 1989). The amplified fragments were cut with relevant restriction enzymes, and then inserted into the MCS (multiple cloning site) of pCEA, resulting in the expression vector, pCEAc-dtk. Between the cd and tk, there was a linker which encoded ten glycines and one serine.

B. Cell culture and transfection

The cell lines, LoVo, HT-29 (human colon carcinoma) and GLC-82 (human lung adenocarcinoma), SGC-7901 (human stomach carcinoma), BEL-7402 (human hepatoma) were used. LoVo, HT-29 (ATCC) and other cell lines (provided by the first Military Medical University and Experimental Animal Center, Sun yat-sen University of Medical Sciences) were cultivated in RPMI 1640 (GIBCO-BRL) medium with 10% fetal calf serum,

100 units/ml penicillin and $100 \mu\text{g/ml}$ streptomycin. No mycoplasma was detected by PCR. Cells were transfected with pCEAc-dtk by use of ESCORT transfection reagent (Sigma), and the positive clones were selected with G418(GIBCO-BRL) for fourteen days. These cells were used for measuring the enzymatic activities of *E. coli* CD and HSV1-TK, and for cytotoxicity assay.

C. Enzymatic activities of CD, TK and cytotoxicity assay

E. coli CD activity was measured according the method described (Austin & Huber, 1993). The buffer was 50 mM Tris-HCl (pH7.3), 0.5 mM cytosine (Sigma). Specific activity was defined as nmol of cytosine deaminated/min/mg proteins. The molar extinction coefficient was 1.038×10^3 litre/mol/cm. TK activity was detected as follows: 25 μl of cell extract, 75 μl of reaction buffer contained 50 mM Tris-HCl (pH7.5), 10mM ATP, 10 mM MgCl₂, 10 mM β -mercaptoethanol, 10 mM NaF, 50 $\mu\text{g/ml}$ PMSF(Sigma) and $2 \mu\text{mol/L}$ ³HdT (20Ci/mmol). The mixture was incubated at 37 °C for 30 min, and then 100 μl of reaction mixture was dropped onto DE-81 filter paper (Whatman). The paper was washed with 95% ethanol three times, and then put in 5-ml scintillation liquid for measuring CPM. The relative activity of TK was defined as follows:

$$\frac{\text{CPM/mg of proteins in the cells transfected with pCEAc-dtk}}{\text{CPM/mg of proteins in the cells transfected with pcDNA3tk}} \times 100\%$$

In pcDNA3tk, the CMV promoter drove the expression of HSV1-tk gene cut from pHSV106 with BglIII and EcoRI.

The cytotoxicity assay was carried out by MTT (Sigma) assay. In a 96-well culture plate, 2×10^3 cells/well were seeded,

and the different concentrations of 5-FC (Sigma) and GCV (Roche) were added. After 72 hrs, 10 μ l of MTT (5 mg/ml) was added into each well and incubated in 37 °C for 4hrs. The supernatant was discarded and 150 μ l/well of DMSO was added. The absorbance (A) was measured at 570 nm. The survival rate = $A_{\text{treated}}/A_{\text{control}} \times 100\%$. The experiment was performed three times.

D. Radiosensitization

The sensitivity of tumor cell expressing CD and TK was carried out according to the method described by Price & McMillan(1990). An X-ray instrument was used, and the dose rate was 106.82 cGR/min. The surviving fraction was calculated as follows:

(Cell number in the control well) divided by (Cell number in the radiated well or prodrug/radiation-treated well) $\times 100\%$. The data and charts was processed and produced by the Department of Radiobiology, Tumor Hospital of China Academia.

E. In vivo studies

GLC-82 cells transfected with pCEAc-dtk were inoculated subcutaneously into 6-8 wk BALB/C-nu/nu mice, and tumors were allowed to grow for about one month. Afterwards, the tumor tissue was surgically removed and cut into 1-mm size fragments which were implanted under the renal capsules of BALB/C-nu/nu mice. 5-FC and GCV were delivered by intraperitoneal injection at days 2 and 3. 10 days later, the animals were sacrificed. The tumor volume = $(a \times b^2) / 2$ (mm^3), where a is: the longest diameter of the tumor, and b: the shortest diameter. The tumor inhibition rate was calculated as follows:

$$\frac{(Dn - Do_{\text{in control}}) - (Dn - Do_{\text{in subject}})}{Dn - Do_{\text{in control}}} \times 100\%$$

Do: the tumor volume before translated into subrenal capsule; Dn: the tumor volume after the mouse was sacrificed.

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