Cre-LoxP Mediated Strong Enhancement of pBIRC5 Promoter Driven Suicide of Cancer Cells with CD/UPRT and Fluorocytosine

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Abstract: Suicide gene therapy (gene-directed enzyme prodrug therapy, GDEPT), based on tumor-specific promoter driven expression of genes encoding an enzyme capable of intracellularly converting a extracellularly administered non-toxic prodrug into a toxin that selectively kills cancer cells, is a promising approach for cancer treatment. An important prerequisite for the practical use of the approach is cancer-specific suicide gene expression. Since many tumor specific promoters used for intratumoral expression of suicide genes are relatively weak, it was suggested to use tumor specific expression of Cre recombinase to enhance the expression of the therapeutic transgene driven by a strong promoter, artificially suppressed with a floxed insertion, but reactivated by the excision of this insertion with Cre.

In this report, we demonstrate that the expression level of the chimeric suicide gene (FCU1), encoding a cytosine deaminase (CD)/uracil phosphoribosyltransferase (UPRT) fusion protein, under control of the cancer-specific human pBIRC5-1.5 promoter, as well as its cytotoxicity in the presence of 5-FC in various cell lines, is essentially lower than under control of the strong ubiquitous pCMV promoter. However, the use of the binary system including Cre enzyme expressed under control of the pBIRC5-1.5 promoter and the FCU1 driven by pCMV connected to the gene via a floxed transcriptional stop signal allows to increase the cell specific expression level and cytotoxicity of CD/UPRT up to the values comparable to those achieved with the pCMV promoter immediately adjacent to FCU1. Thus, this combination may be useful for human gene therapy applications.

Keywords: Gene therapy, bystander effect, cytosine deaminase, survivin, uracil phosphoribosyltransferase.

INTRODUCTION

Though still having problems in clinical trials, one of the seemingly most promising strategies for cancer gene therapy is gene-directed enzyme prodrug therapy (GDEPT) [1]. In this therapy, a gene (called suicide or therapeutic gene) encoding an enzyme is introduced into tumor cells, followed by administration of a prodrug, which is intracellularly converted by the expressed enzyme to a cytotoxin, killing the cells. [2 (mini-review)]. Such a therapy is especially advantageous in that if even only a small portion of cancer cells contain the prodrug and express the suicide gene, the toxic products of the prodrug conversion spread to neighboring cancer cells and kill them. This bystander cell killing (bystander effect) may greatly improve the effectiveness of cancer therapy. Numerous studies performed in animal models with a variety of enzyme encoding gene/prodrug combinations have demonstrated that complete tumor eradication is possible even when the suicide gene product is expressed by approximately 10% of the tumor cells [3]. The first GDEPT system described [4, 5] was the thymidine kinase gene of the Herpes Simplex virus (HSVtk) combined with ganciclovir (GCV) as the prodrug. This system showed very promising results in animal models with various tumors [6], but the success was much more modest in numerous clinical trials (for reviews, see [2, 7, 8]).

Clearly, a number of factors are responsible for this phenomenon, and there are numerous suggestions how to eliminate such a discrepancy. One of the most obvious potential solutions to this problem is to enhance the bystander effect, which could be achieved by increasing the toxin production in cancer cells. However, known tumor specific promoters are relatively weak, thus not allowing high level toxin production. On the other hand, known strong promoters such as pCMV are not tissue specific and their use makes the therapy more hazardous. To solve this problem, a novel approach have been developed, in which the therapeutic gene is controlled by a suppressed strong tumor-nonspecific promoter capable of being activated by an enzyme expressed in the same cell from a gene under control of a cancer-specific promoter. Such a binary system might allow to achieve strong and at the same time cancer-specific expression of the therapeutic gene, and thus to potentiate the effectiveness of suicide gene therapy. One of possible implementations of this system is the use of the strong pCMV promoter silenced by a floxed transcriptional stop signal linking the promoter to the therapeutically gene. If the Cre gene is expressed in the same cells under control of a weak cancer-specific human pro-
moter, the pCMV promoter is reactivated due to the excision of the stop signal by Cre recombinase.

Quite a few tumor-specific promoters were tested in similar systems (for review, see [9-16]. One of the most promising promoters, which is active in the majority of cancers but not in normal cells, seems to be the pBIRC5-1.5 promoter of the BIRC5 gene encoding an apoptosis inhibitor-survivin. BIRC5 is expressed in many cancers but not in normal adult tissues, and its expression is mainly regulated transcriptionally [17-20]. There are several reports supporting the feasibility of using the pBIRC5-1.5 promoter to induce cancer-specific expression of transgenes or viral vectors in cancer gene therapy [17, 21-25]. It was reported that the pBIRC5-1.5 promoter was over 200 times more cancer-specific than the cytomegalovirus promoter pCMV in vivo [21], although there was also a contradictory report [26]. In general, the reported results confirm a high cancer-specificity of the pBIRC5-1.5 promoter. Despite this advantageous feature, the pBIRC5-1.5 promoter was surprisingly rarely used for GDEPT [12, 15]. As far as we know, there are only two reports describing the pBIRC5-1.5 promoter used for mammalian expression vector pCI (Promega) to give pCI-pCMV-FCU1 vector. The HindIII-NotI fragment containing the FCU1 gene was excised, its NotI site filled in with Klenow fragment, and the resulting fragment cloned into pGL3-pCMV (Promega) to give pGL3-pCMV-FCU1 vector further used as positive control. pGEM-T/pBIRC5-1.5 vector carrying a ~1456 to +42 bp fragment (pBIRC5-1.5) of a ~1500-bp BIRC5-1.5 promoter region, flanked by BgIII and NotI sites, was designed in our laboratory earlier. The BgIII-NotI sequence containing pBIRC5-1.5 from pGEM-T/pBIRC5-1.5 was cloned into the corresponding sites of pGL3-Basic vector (Promega) to create pGL3-pBIRC5-1.5 vector. The luciferase gene (luc+) of pGL3-pBIRC5-1.5 plasmid was substituted by the FCU1 fusion gene from pCI-pCMV-FCU1 to give the final construct named pGL3-pBIRC5-1.5-FCU1 (Fig. 1a).

pQXIX-Cre vector was previously generated by incorporating a NotI-EcoRI fragment of pCre vector (kindly provided by Dr. A. Fradkov), that contained a 1053-bp fragment of the Cre recombinase gene, into the corresponding sites of pQXIX (Clontech). To create pQXIX-pBIRC5-1.5-Cre vector, a BgIII-NotI fragment of pGEM-T/pBIRC5-1.5 was cloned into the corresponding sites of pQXIX-Cre. The pQXIX-pBIRC5-1.5-Cre vector was used as a Cre recombinase donor in the binary system (Fig. 1a).

Cre-regulated FCU1 expression vector, pGL3-pCMV-LoxP-Stop-LoxP-FCU1, comprised a 743-bp fragment of the cytomegalovirus immediate-early enhancer/promoter region separated from the FCU1 gene by a LoxP-flanked stuffer DNA containing three tandem repeats of the SV40 late polyadenylation signal from pGL3-Basic (Fig. 1a).

For negative control, we constructed pGL3-(no promoter)-FCU1. The pBIRC5-1.5 sequence was removed from pGL3-pBIRC5-1.5-FCU1 plasmid using the Smal and HindIII enzymes. The ends were made blunt with Klenow fragment, and the vector was religated.

Cell Cultures

The following cancer cell lines were used: transformed human kidney cells (HEK 293), epidermoid lung carcinoma (Calu1), lung carcinoma (A549), and human fibrosarcoma cells (HT1080). The cells were grown in DMEM/F12 (1:1) medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (Invitrogen) at 37°C and 5% CO2. All cell lines were tested before use and found to be free of Mycoplasma infection.
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Fig. (1). Expression of the FCUI gene in human cancer cells. (a) Scheme of plasmid constructs used in this study. pGL3-pCMV-FCU1, vector expressing FCU1 under control of the strong constitutive pCMV promoter; pGL3-pBIRC5-1.5-FCU1, vector expressing FCU1 under control of the cancer specific pBIRC5-1.5 promoter; pGL3-(no promoter)-FCU1, promoterless vector carrying FCU1. Promoters and the FCU1 gene are denoted by empty arrows and empty rectangles, respectively, intron is denoted by V shape line; (b) western blot detection of FCU1 expression by different constructs in transfected human cancer cells. p53 - (mutated) and p53+ (wild type) show the p53 status.

Cell Transfection

Cells were transfected in 25 cm² Corning flasks or 6-well plates with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations.

Measuring Transfection Efficiency

To determine transfection efficiency, reporter plasmids, carrying the reporter GFP gene driven by the pCMV immediate early promoter, were used. Cells were transfected in 6-well plates with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. 48 hours post-transfection, cells were photographed on a Nikon fluorescence microscope (200 x field) at 395-nm excitation, and the number of fluorescent cells was determined in 10 microscopic fields. To determine the total number of cells, the cells were stained with Hoechst 33258 and counted by fluorescence microscopy at 350-nm excitation. The percentage of transfected cells was counted by comparing the number of cells expressing the reporter protein to the total number of cells in the population.

Western Blot Analysis

Cells were lysed in SDS sample buffer, and proteins were separated by 100 g/L SDS-PAGE and transferred to PVDF membranes. To visualize cytosine deaminase, a sheep polyclonal antiserum directed against yeast CD (Abcam) and goat anti-sheep IgG-horseradish peroxidase conjugates (Promega) were used. Detection of reactive bands was facilitated by using a horseradish peroxidase-linked secondary conjugate and ECL detection reagents (Biorad). Cre recombinase was determined with mouse monoclonal antibody (Abcam) and donkey anti-mouse IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology). GAPDH was determined with mouse monoclonal antibody (Santa Cruz Biotechnology) and donkey anti-mouse IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology).

In Vitro Cell Sensitivity to 5-FC and 5-FU

Human tumor cells were transiently transfected with the generated vectors. A total of (1.5-2)×10⁶ cells/well were plated onto 6-well culture dishes in 2 ml of medium. The next day, the cells were seeded in triplicate in 96-well plates (Becton Dickinson) at a density of 2×10³ cells/well in 200 µl of medium. Then, 100 µl of medium supplemented with serial dilutions of 5-FC (for transfected cells) or 5-FU (in case of intact human tumor cells) (0–1000 µM) was added. Medium with 5-FC (5-FU) was refreshed every 48 hr for a further 120 hr culture period. The number of viable cells was counted by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay according to the protocol (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). The results were expressed as a ratio between the number of viable cells in plates containing the drugs and the number of viable cells in the corresponding drug-free controls. Three independent transfections were performed for each experimental construct. The IC₅₀ values of 5-FC or 5-FU were calculated using a curve-fitting parameter, and the results were represented as mean±SD of three independent experiments.

Statistical Analyses

Statistical analysis was performed using GraphPad PRISM 5.0 software (GraphPad Software Inc), and a value of P<0.05 was considered statistically significant. The data were represented as mean±SD of three independent experiments.

RESULTS

1. Bifunctional Yeast Cytosine Deaminase/Uracil Phosphoribosyltransferase Fusion Gene as the Therapeutic Gene

In this study, we used the FCY1-FUR1 (FCU1) fused gene as a suicide gene, earlier reported to exhibit a high efficiency of 5-FC intracellular prodrug conversion into its cytotoxic form 5-FU and provide a stronger bystander effect.
in vitro and in vivo [30, 31]. Its fusion protein CD/UPRT deaminates 5-FU and converts 5-FU into 5-fluoro-UMP (FUMP). This conversion finally results in cellular accumulation of 5-FUTP and 5-fluoro-dUMP (5-FdUMP). 5-FUTP incorporates into DNA thus inhibiting nuclear processing of ribosomal and messenger RNAs, while 5-FdUMP irreversibly inhibits thymidylate synthase preventing DNA synthesis [28]. Earlier it was shown that tumor cells transduced with an adenosine expressing the FCU1 fusion gene were sensitive to concentrations of 5-FU 1000-fold lower than cells transduced with a vector expressing only FCY1 [28]. We constructed the fusion gene by joining a 474 bp fragment of FCY1 (cytosine deaminase gene) to a 645 bp fragment of FUR1 (uracil phosphoribosyltransferase gene) as described in a previous report [29]. The stop codon of FCY1 and start codon of FUR1 were removed by oligonucleotide-directed mutagenesis (see Materials and Methods). The two gene moieties were joined in-frame resulting in two ORFs linked via an Ala residue.

2. Vectors for Comparative Study of the pCMV and pBIRC5-1.5 Promoter Efficiencies in Expression of FCU1

Fig. (1a) schematically shows the vectors harboring the FCU1 gene under direct control of promoters.

Vector pGL3-pCMV-FCU1 contained a 743-bp fragment of the cytomegalovirus immediate-early enhancer/promoter region separated from the 1119-bp long FCU1 gene by a 133-bp chimeric intron taken from pCI vector (Promega) (Fig. 1a). Earlier it was demonstrated that the presence of an intron flanking the cDNA insert frequently increases the level of gene expression [32-35].

We used a -1456 to +42 bp promoter containing fragment of BIRC5-1.5 (designated as pBIRC5-1.5) as a cancer-specific promoter [27, 36] and cloned the gene under its control in pGL3-pBIRC5-1.5-FCU1 (Fig. 1a), where the suicide gene and promoter were separated by a 133-bp chimeric intron identical to that in pGL3-pCMV-FCU1.

For negative control, we constructed a pGL3-(no promoter)-FCU1 plasmid by completely removing the pBIRC5-1.5 sequence from pGL3-pBIRC5-1.5-FCU1 (Fig. 1a).

3. The Efficiencies of the pBIRC5-1.5 and pCMV Promoters in Direct Expression of FCU1 Gene

Fig. (1b) shows the Western blot analysis of the FCU1 gene expression level in three cell lines: HEK293 (p53-transformed human kidney cells), CaluI (p53’ epidermoid lung carcinoma) and A549 (p53’ lung adenocarcinoma) cells were transiently transfected with pGL3-pBIRC5-1.5-FCU1, pGL3-pCMV-FCU1, or pGL3-(no promoter)-FCU1 as a negative control. Cell lysates were analyzed by Western blotting with an antibody against the CD protein 48 h after transfection. In all the cell lines tested, the pGL3-pCMV-FCU1 vector produced a higher and p53 status-independent level of the CD/UPRT protein compared with that for pGL3-pBIRC5-1.5-FCU1. The FCU1 expression in A549 was detected only when transfected with pGL3-pCMV-FCU1. As judged from the absence of the CD/UPRT protein in cells transfected with the promoterless vector, the latter had no cryptic promoter activity. Thus, the pBIRC5-1.5 promoter was capable of directing cell specific FCU1 expression, however, less efficiently than the strong constitutive pCMV promoter, in agreement with the reports on the relative weakness of pBIRC5-1.5 [27].

To evaluate the cytotoxic potential of pGL3-pBIRC5-1.5-FCU1, we transiently transfected HEK293 and A549 cells with this vector and tested their survival in the presence of 5-FC. Fig. (2) shows that the cytotoxicity of pGL3-pCMV-FCU1 was significantly higher than that of pGL3-pBIRC5-1.5-FCU1. Under the conditions used, the survival of HEK293, CaluI, and A549 cells transfected with pGL3-pCMV-FCU1 was about the same: 15, 18 and 22%, respectively. The survival of the same cells observed after transfection with pGL3-pBIRC5-1.5-FCU1 was 32, 48 and 86%, respectively. These data also demonstrate that although the pBIRC5-1.5 promoter is less efficient than pCMV, it is highly cell specific (no cytotoxic effect in A549 cells).

![Fig. (2) 5-FC sensitivity of HEK293, CaluI, and A549 tumor cells transfected with pGL3-pCMV-FCU1, pGL3-pBIRC5-1.5-FCU1 or pGL3-(no promoter)-FCU1 constructs. The height of histogram bars corresponds to the percentage of survived cells with respect to those for control without 5-FC treatment and represents mean of at least three independent experiments; the error bars indicate standard deviation.](image)

In control experiments, 500 µM 5-FC had a much lesser effect on nontransfected cells: all the cell lines tested showed more than 85% survival.

4. Design of a Cre-LoxP//pCMV-Stop-FCU1 System for Enhancement of Cell-Specific FCU1 Expression and Analysis of the System’s Efficiency

To enhance the level of FCU1 expression while maintaining its cancer specificity, we used the Cre-LoxP//pCMV-Stop-FCU1 system [38-41]. Fig. (3) illustrates the design of the system used in our study:
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Fig. (3). Schematic illustration of the modified Cre-LoxP/pCMV-Stop-FCU1 system action. Cre recombinase, expressed by the pBIRC5-1.5 promoter, excises the ‘stop sequence’ from the pGL3-pCMV-LoxP-Stop-LoxP-FCU1 vector and thus activates the FCU1 gene expression driven by the strong pCMV promoter. The ‘stop sequence’ is a 705-bp triple repeat of the 235-bp SV40 late polyadenylation signal. NLS is a nuclear localization signal. Promoters and the FCU1 gene are denoted by empty arrows and empty rectangles, respectively; filled arrows denote LoxP sequences; the stuffer (‘Stop’) sequence is marked as a blank circle.

(i) The pQXIX-pBIRC5-1.5-Cre plasmid expresses Cre recombinase tagged with a nuclear localization signal (NLS) under control of the pBIRC5-1.5 promoter (confirmed by Western blots, data not shown).

(ii) The vector carrying the suicide gene contains a stuffer sequence of three tandem SV40 polyadenylation signals between the strong constitutive pCMV promoter and the FCU1 gene. This stop signal blocks the expression of FCU1. It is flanked by 34-bp LoxP sites recognized by Cre recombinase. This construction will be denoted below as LoxP-Stop-LoxP. Excision of the stop signal by Cre was supposed to trigger high-level expression of FCU1 under the pCMV promoter.

Fig. (4) shows the efficiency of the Cre-LoxP/pCMV-Stop-FCU1 system in expressing FCU1 analyzed by Western blotting. As seen from the figure, the stop signal used completely blocked transcription of FCU1. A double transfection with suicide and Cre vectors provided the expression level of FCU1 comparable to that in cells transfected with pGL3-pCMV-FCU1, wherein the pCMV promoter was directly connected to FCU1.

5. Enhancement of Tumor Cell Sensitivity to 5-FC by co-Transfection of Expression Vectors Carrying pBIRC5-1.5-Cre and pCMV-LoxP-Stop-LoxP-FCU1 Constructs

To analyze the effect of the Cre-LoxP/pCMV-Stop-FCU1 system on tumor cell sensitivity to 5-FC prodrug, the cells (listed in Table 1) were transfected with 5:5 μg of pQXIX-pBIRC5-1.5-Cre and pGL3-pCMV-LoxP-Stop-LoxP-FCU1 or pGL3-pBIRC5-1.5-FCU1 and pGL3-pCMV-FCU1 (as a positive control). Cells were cultured for 5 days in medium containing various concentrations of 5-FC: 0, 10, 50, 200, 500 and 1000 μM. The ratio and quantity of the two vectors used for the transfections were optimized in a preliminary study (data not shown).

Table 1 shows the IC_{50} values (the prodrug concentration that provides 50% inhibition of cell growth) for 5-FC in tested cell lines. A double transfection made p53 negative CaluI and HEK293 cells almost 16- and 5-fold, respectively, more sensitive to 5-FC than a transfection with just pGL3-pBIRC5-1.5-FCU1 alone, thus demonstrating high efficiency of the system in these cells. In contrast, IC_{50} values in p53 positive A549 and HT1080 cells could not be determined due to very low cell death rates. It is in line with the suppressive effect of p53 on pBIRC5-1.5 promoter activity. Interestingly, A549 cells were considerably more resistant to 5-FC than other cells when FCU1 was expressed under direct pCMV control.

6. The Cre-LoxP/pCMV-Stop-FCU1 System Provides the pBIRC5-1.5 Promoter Specificity of the FCU1 Gene Expression

The Cre-LoxP/pCMV-Stop-FCU1 system described above was tested in cells with wild-type p53 status, such as A549 and HT1080. Recent studies demonstrated that transfection with wild-type p53 was associated with an almost full inhibition of pBIRC5-1.5 promoter activity. It was found that this promoter contains two p53 binding sites apparently not essential for transcriptional inactivation [37]. Previously, it
was demonstrated that p53 may act as a transcriptional repressor by recruiting the histone deacetylase (HDAC) complex and that HDAC may be involved in p53 dependent repression of BIRC5-1.5 expression [37]. Moreover, it was shown that down-regulation of the BIRC5-1.5 gene is mediated by p53 through recruitment of DNA cytosine methyltransferase to its promoter [42]. Therefore, modification of the pBIRC5-1.5 promoter chromatin region might be a molecular explanation for silencing BIRC5-1.5 gene transcription by p53.

Strong cytotoxic effects in HT1080 and A549 cells were observed only after transfection with pGL3-pCMV-FCU1 (Fig. 4, Table 1). Transfection of these cells with pGL3-pBIRC5-1.5-FCU1 or double transfection with pGL3-pBIRC5-1.5-Cre and pGL3-pCMV-LoxP-Stop-LoxP-FCU1 induced no cytotoxicity. It is worth mentioning that the expression level of FCU1 under the pCMV promoter and cytotoxicity with 5-FC in A549 cells were considerably lower than in other cells (Table 1, Fig. 4). There were reports [43] that the pCMV promoter possesses a higher activity in HEK293 cells as compared with A549. We tested the possibility of lower transfection efficiency in the case of A549 as compared to HEK293 cells. To this end, we transfected each cell line with plasmids containing the GFP reporter gene under control of the pCMV promoter and determined the proportion of fluorescent cells. We found that as little as 4% of the A549 cells versus 26% of the HEK293 cells had contained GFP plasmid. Therefore, transfection of the A549 cells is indeed less efficient, but it remains unclear if it can explain all the difference in cytotoxicity.

**DISCUSSION AND CONCLUSION**

Cancer-specific expression of a therapeutic gene has emerged as a potentially fruitful approach for cancer gene therapy that could increase the safety of this therapy by confining the cytotoxic effect to tumor cells. A number of cancer-specific promoters have been reported, such as those of probasin, human telomerase, reverse transcriptase, survivin, ceruloplasmin, HER-2, osteocalcin, and carcinoembryonic antigen (reviewed in [9, 37, 44]). Studies on animal models showed that cancer-specific promoters had a clear advantage in targeting cytotoxicity to cancer cells compared with strong constitutive promoters like the human pCMV promoter often used in clinical trials [37].

Effective use of a cancer-specific promoter largely depends on four features: (i) in what types of tumors the promoter can direct expression of the desired gene (tumor “universality”) [11, 27]. (ii) in what portion of tumors of a given type the promoter is active (tumor polymorphism regarding the promoter activity) [45], (iii) the promoter strength in tumor cells, and (iv) tumor specificity of the promoter [26], [27].

An ideal cancer-specific promoter should be universal to work in all types of tumors and in all cells of a given tumor type, and it should be strong and active only in tumors and not in normal tissues (“tumor-on/liver–off” principle [17]). Such an ideal promoter has not been found so far, although attempts to construct chimeric promoters with improved properties have been reported [46]. It quite may be that cancer-specific universal promoters do exist in nature, however, only a very small proportion of all promoters deposited in the corresponding databases were tested in gene therapy experiments (e.g. see CAPRIS or TIPROD databases). Therefore, it remains to choose a known promoter as much as possible approaching to the ideal. As discussed in [17], one of the attractive candidates for this role is the pBIRC5-1.5 promoter.

The survivin protein is known to be expressed in most tumor cell types but not in the overwhelming majority of adult normal cells [47, 48]. Survivin mRNA or protein overexpression was demonstrated in tumors of the lung, breast, colon, ovaries, skin, melanoma etc. [17, 49]. Recent studies have demonstrated the ability of the human pBIRC5-1.5 promoter to direct specific expression of transgenes in lung and breast cancers, glioma and other malignant tissues [17, 44, 50-54]. According to these data, the pBIRC5-1.5 promoter is highly tumor specific [27, 45, 49]. As to the universality of the promoter, the level of survivin is elevated in 63% of tumor samples from patients with lung cancer and in 43% of tumor samples from patients with esophageal cancer. This only partial upregulation is probably due to general

### Table 1. 5-FU Sensitivity of Non-Transfected Cells and 5-FC Sensitivity of Transfected Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>p53 Status</th>
<th>IC₅₀ of 5-FU, µM</th>
<th>Transfected Cells: IC₅₀ of 5-FC, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pGL3-pCMV-FCU₁</td>
</tr>
<tr>
<td>HEK293</td>
<td>mut</td>
<td>13±7</td>
<td>50±9</td>
</tr>
<tr>
<td>Calu</td>
<td>mut</td>
<td>110±18</td>
<td>240±10</td>
</tr>
<tr>
<td>HT1080</td>
<td>wt</td>
<td>4±2</td>
<td>233±13</td>
</tr>
<tr>
<td>A549</td>
<td>wt</td>
<td>900±204</td>
<td>946±180</td>
</tr>
</tbody>
</table>

Tumor cells were transfected with constructs indicated in the three rightmost columns. The next day after transfection, medium was changed with fresh medium supplemented with serial concentrations of 5-FC (0–1000 µM). The cells were then cultured for 5 days, with medium changed every other day. Cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. IC₅₀ values for 5-FC were calculated using a curve-fitting parameter. The results are represented as mean ±SD of three independent assays.

mut, mutant; p53

wt, wild type p53

ND, not determined
tumor polymorphism regarding any certain gene expression. Such a tumor dependent polymorphism [55-57], is due to the inherent property of all tumors – their heterogeneity [58]. A similar polymorphism was reported for the expression of the BIRC5 gene in non-small-cell lung carcinoma [21]. Other types of cancers also displayed various levels of the polymorphism (e.g. see [49]). It makes necessary to analyze BIRC5-1.5 expression in tumor specimens of cancer patients to identify those patients who might benefit from gene therapy using the pBIRC5-1.5 promoter. This feature prompts to use combinations of different promoters to make the therapy more universal.

Another problem with all known cancer-specific promoters is their relative weakness [16, 46]. However, this disadvantage can be overcome, and at least two systems were reported to be suitable for this goal: the described above Cre-LoxP system combined with a silenced strong constitutive promoter [59], and a HIV1 tat-tar combination allowing to increase the rate of transcription under control of a cancer-specific promoter [12].

In this study, we demonstrate the potential of the Cre-LoxP/pCMV-Stop-FCU1 system to enhance pBIRC5-1.5 promoter controlled cancer-specific expression of one of the most promising suicide gene FCU1 capable of killing tumor cells via 5-FC. This system has a number of important advantages: yeast cytosine deaminase is more thermostable than the bacterial enzyme [8]; expression of the FCU1 gene makes cells 1000-fold more sensitive to 5-FC than cytosine deaminase alone [28]; the system seems to be more efficient than the HSVtk/GCV system, because it does not need direct cell-to-cell contact to transport toxic metabolites to neighboring cells through gap junctions [28, 60]. In addition, as opposed to the HSVtk/GCV system, the FCU1/S-FC system is efficient in both dividing and nondividing cancer cells [5].

Despite clear success in murine models and in vitro (for recent reports, see [29, 60, 61]), clinical trials with the FCU1 gene were rather disappointing [62]. This might be due to low suicide gene expression and, consequently, insufficient bystander effect.

Therefore, we tried to find the ways of enhancing the suicide FCU1 gene cancer-specific expression. In the course of our experiments, we have made a number of significant findings.

1. The expression of the fusion gene under pBIRC5-1.5 control and its capacity to kill tumor cells strongly depends on the p53 status of cells. This phenomenon was described earlier by us and other authors for other genes under the pBIRC5-1.5 promoter [37, 63]. Accordingly, the cytotoxic effect of 5-FC is considerably lower in p53+ (A549 and HT1080) than in p53- cells (Calu1 and HEK293). This effect is in agreement with our previous study [27] and is due to the known inhibition of pBIRC5-1.5 by p53 [63]. Despite the fact that p53 is damaged in only about 50% of cancers, an enhanced survivin level is observed in a vast majority of tumors. This contradiction remains unresolved so far. Certain tumors lacking p53 mutations are known to show high level of survivin, suggesting that there are a number of signaling pathways that contribute to the expression of its gene. For example, suppression of BIRC5 transcription by p53 was reported to be inhibited through the interaction of Estrogen Receptor α with p53 [50]. Moreover, it was demonstrated that knockdown of Retinoblastoma protein suppressor (upregulated in tumor cells) resulted in increased levels of survivin irrespective of tumor p53 status [64]. It was reported that murine double minute oncogene, which is over-expressed in many tumors [65], could promote p53 degradation and suppress nuclear export of p53 thus preventing interaction of p53 with pBIRC5-1.5 [50, 64, 66]. One can also suggest the existence of unidentified cis-regulatory elements outside the pBIRC5-1.5 sequence that make BIRC5-1.5 expression independent on p53 status in cancer cells in vivo. Identification of these elements and their use in expression systems might considerably improve the applicability of pBIRC5-1.5 for GDEPT. But GDEPT using the pBIRC5-1.5 promoter can be already now applied for treatment of tumors with defective p53 which are common in lung cancer and many other tumors, ranging from 33% in adenocarcinomas to 70% in small cell lung cancers [48, 67-69]. Treatment of most of them is still far from success [70].

2. We showed that, like in controlling other genes [26, 71], the human pBIRC5-1.5 promoter in permissive p53- cells ensured significantly lower levels of transcription and cytotoxicity of the FCU1 gene than the strong constitutive pCMV promoter (Table I).

3. Different cells transfected with FCU1 under the pCMV promoter control show different levels of sensitivity to 5-FC: HEK293 (p53+)>Calu1 (p53-) ≈ HT1080 (p53-) > A549 (p53-). A similar order of sensitivity to 5-FC was observed in permissive p53- cells for pBIRC5-1.5 directed FCU1 expression: HEK293 (p53+)>Calu1 (p53-). The ratio of the 5-FC sensitivity levels for these cells is approximately the same for pCMV and pBIRC5-1.5 promoters: IC50 (HEK293)/IC50 (Calu1)=0.2. In the Calu1, A549 and HT1080 cells, the relative pBIRC5-1.5 and pCMV promoter activity, measured by Western blotting, GFP expression or 5-FC cytotoxicity, correlates with the activity measured by a luciferase assay [27].

4. For the FCU1/S-FC system with pBIRC5-1.5/Cre-LoxP mediated activation of the silenced pCMV promoter, we observed the same qualitative trends in the expression and cytotoxicity levels as in the case of FCU1 under direct pBIRC5-1.5 control (Table 1, Fig. 4): expression and cytotoxicity were detected only in p53- cells, and HEK293 (p53-) cells were more sensitive to 5-FC than Calu1 (p53-). In permissive cells, the levels of expression and cytotoxicity in the case of Cre induced pCMV-LoxP-Stop-LoxP-FCU1 were considerably higher, being increased up to 5- and 16-fold for HEK293 and Calu1 cells, respectively, as compared to pBIRC5-1.5-FCU1. However, the expression level was still lower than that for the pCMV promoter immediately adjacent to FCU1. There may be several reasons for this effect. The first, and most probable, is that simultaneous penetration of Cre- and FCU1-carrying vectors into one and the same cell is less probable than penetration of a single vector. The second is that the excision of the stuffer DNA from the killer vector by Cre recombinase may be incomplete. Finally, the CD/UPRT accumulation in cells transfected with pCMV-FCU1 may be faster than that in cells transfected with pCMV-LoxP-Stop-LoxP-FCU1 and the Cre carrying vector,
because it does not require additional steps of Cre recombinase synthesis and stop signal excision. Also, the presence of a LoxP sequence retained in the CD-UPRT-producing construct after excision of the stop signal by Cre may have negative effect on the FCU1 expression.

In any case, the expression enhancement we achieved is significant, and the Cre-LoxP mediated tumor-specific expression of the FCU1 suicide gene under the pCMV promoter, described here, may be helpful in improving the efficiency of the FCU1 application for gene therapy purposes.

**ABBREVIATIONS**

5-FC = 5-fluorocytosin
5-FU = 5-fluorouracil
FCU1 = chimeric gene obtained by splicing of the yeast FCY1 gene encoding cytosine deaminase (CD) and the FUR1 gene encoding uracil phosphoribosyltransferase (UPRT)
pBIRC5 = 1.5 - 1.5-kb promoter of the human BIRC5 gene encoding survivin
pCMV = human cytomegalovirus (CMV) promoter

**CONFLICT OF INTEREST**

There is no conflict of interest.

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