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Targeted cytosine deaminase-uracil phosphoribosyl transferase suicide gene therapy induces small cell lung cancer specific cytotoxicity and tumor growth delay

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Abstract

Purpose—Small cell lung cancer (SCLC) is a highly malignant cancer for which there is no curable treatment and novel therapies are therefore in high demand. In the present study we investigated the therapeutic effect of transcriptionally targeted suicide gene therapy for SCLC based on the yeast cytosine deaminase (YCD) gene alone or fused with the yeast uracil phosphoribosyl transferase (YUPRT) gene followed by administration of 5-fluorocytosine (5-FC) prodrug

Experimental design—The YCD gene or the YCD-YUPRT gene was placed under regulation of the SCLC-specific promoter Insulinoma-associated 1 (INSM1). Therapeutic effect was evaluated *in vitro* in SCLC cell lines and *in vivo* in SCLC xenografted nude mice using the non-viral nanoparticle, DOTAP:Cholesterol for transgene delivery.

Results—INSM1-YCD/5-FC and INSM1-YCD-YUPRT/5-FC therapy induced high cytotoxicity in a range of SCLC cell lines. The highest therapeutic effect was obtained from the YCD-YUPRT fusion gene strategy. No cytotoxicity was induced after treatment of cell lines of other origin than SCLC. In addition the INSM1-YCD-YUPRT/5-FC therapy was superior to an established suicide gene system consisting of the Herpes Simplex Virus Thymidine Kinase (HSVTK) gene and prodrug Ganciclovir (GCV). The superior effect was in part due to massive bystander cytotoxicity of YCD-YUPRT-produced toxins. Finally, INSM1-YCD-YUPRT/5-FC therapy induced significant tumor growth delay in SCLC xenografts compared to control treated xenografts.

Conclusions—The current study is the first to test cytosine deaminase-based suicide gene therapy for SCLC and the first to demonstrate an anti-tumor effect from the delivery of suicide gene therapeutics for SCLC *in vivo*.

Keywords

gene therapy; transcriptional targeting; Insulinoma-associated 1 promoter; small cell lung cancer; suicide genes

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Introduction

Small cell lung cancer (SCLC) represents nearly 20 % of lung cancer cases and is characterized by a distinct neuroendocrine phenotype, aggressive progression and early metastasis. Most newly diagnosed patients with SCLC respond to first-line chemo- and radiotherapy, but due to a high frequency of treatment-resistant relapse the 5-year survival rate is below 10 %. Despite many efforts to improve prognostics with presently available modalities, there have only been subtle improvements in the survival rate for SCLC patients for the last three decades(1;2). Advancement of novel therapies to replace or complement existing treatment regimes is therefore in high priority for this malignancy.

Suicide gene therapy constitutes a novel promising treatment strategy for cancer, and is based on the introduction of a therapeutic gene encoding an enzyme capable of transforming a non-toxic prodrug into a cell toxin(3). The suicide gene-driven production of toxins obligates strict transgene regulation to obtain activation of prodrug exclusively in malignant cells and avoid toxic side effects. One particular attractive property of suicide gene therapeutics is the ability of produced toxins to spread to nearby cancer cells and induce bystander cytotoxicity. The bystander effect originates from either free diffusion of toxins across the cellular membrane or transport via gap junction intercellular communication (GJIC)(4–6). The combined effect of cancer-specific transgene expression and the local spread of suicide toxins in the tumor environment can mediate efficient drug delivery to a large tumor cell population.

In order to develop transcriptionally regulated suicide gene therapy for SCLC we have previously identified promoters, which confer high and specific regulation of transgenes in SCLC cell lines(7–10). We(9;10) and others(11–16) have previously reported the therapeutic significance of the suicide gene Herpes Simplex Virus Thymidine Kinase (HSVTK) when combined with Ganciclovir (GCV) for SCLC. HSVTK phosphorylates GCV forming GCV mono-phosphate, while the subsequent formation of GCV di- and triphosphates are catalyzed by cellular kinases. GCV triphosphate incorporates into DNA causing replication to be blocked(3). The HSVTK/GCV system has, however, major limitations compromising potential clinical use: i) The highly charged GCV metabolites can only spread to other cancer cells via GJIC, however, since this intercellular communication is greatly compromised in many cancers(17;18), bystander cytotoxicity may be limited accordingly; ii) the incorporation of GCV triphosphates into DNA during replication cause only the actively dividing cell population to be sensitive to treatment(3), and finally iii) GCV is considered a potential carcinogen with adverse systemic toxic effects even at subclinical concentrations(19).

Apart from HSVTK/GCV no other suicide gene therapeutics have been tested for SCLC, although many suicide gene systems with seemingly attractive clinically features have emerged. The suicide gene cytosine deaminase (CD) from either bacteria or fungi converts the clinically safe and non-toxic prodrug 5-fluorocytosin (5-FC)(20) to 5-fluorouracil (5-FU), a clinically approved cytostatic. Cellular enzymes further metabolize 5-FU leading to the formation of active toxic metabolites, which block DNA and RNA synthesis by simultaneous inhibition of the thymidylate synthase and incorporation in DNA and RNA during replication and transcription(3). It has been proven that yeast cytosine deaminase (YCD) has superior catalytic activity compared to its bacterial (*E.coli*) counterpart(21–23) in line with the fact that 5-FC in the clinic is used as an antifungal agent and is less efficient against bacterial infections(20).

To circumvent problems of 5-FU resistance due to increased degradation or poor activation of the toxin(24) introduction of the uracil phosphoribosyl transferase (UPRT) gene in

combination with the CD gene, has been studied. The UPRT enzyme is involved in 5-FU processing in bacteria and yeast and is not present in human cells where other pathways of 5-FU conversion exist. The concomitant expression of the UPRT gene along with CD has been demonstrated to increase therapeutic efficacy markedly(23–27).

Apart from features of safe prodrug application and the ability to affect both the dividing and non-dividing cancer cell population, CD-based suicide gene therapy has shown to induce strong bystander cytotoxicity due to the free diffusion of 5-FU and downstream toxins across cellular membranes(3;4).

In light of these features, we aimed to investigate the therapeutic significance of CD-based therapy for SCLC. The YCD gene alone or fused to the yeast UPRT (YUPRT) gene(23) were cloned for transcriptional regulation from the SCLC specific Insulinoma-associated 1 (INSM1) promoter(8;9). We report that INSM1-regulated YCD-YUPRT/5-FC therapy demonstrate superior cytotoxicity when compared to YCD/5-FC and in particular to HSVTK/GCV therapy in a range of SCLC cell lines and induces massive bystander effects. The clinical relevance of INSM1-YCD-YUPRT/5-FC therapy was further established *in vivo* using a non-viral delivery vehicle DOTAP/Cholesterol (DOTAP/Chol) currently in clinical testing in non-SCLC patients(28). We show that treatment of SCLC xenografted mice with DOTAP/Chol encapsulated INSM1-YCD-YUPRT concomitant with 5-FC administration results in significant tumor growth delay compared to control treated mice.

Materials and methods

Cell lines

The origin, characterization and propagation of all cell lines utilized in the study has been described in detail elsewhere(8;9).

Plasmid vectors

The following plasmid vectors were used: *pGL3 basic* (Promega, Mannheim, Germany). *EGFP-N1* (Clontech, Glostrup, Denmark). *INSM1-HSVtk*: described in Pedersen *et al.*(9). *INSM1-YCD* and *INSM1-YCD-YUPRT*: The YCD and YCD-YUPRT genes were kindly provided by Ulrich M. Lauer (Department of Internal Medicine I, University Clinic Tübingen, Tübingen, Germany) in pLXSN-greenN-YCD and pUC19-SCD (super cytosine deaminase equals YCD-YUPRT) plasmids respectively(23). The excised 0.5 kb YCD (*EcoNI/StuI*) and 1.1 kb YUPRT (*NcoI/HindIII*) fragments were inserted into a INSM1-promoter expression vector (all blunt-end ligations). The plasmid vector was prepared from the excision with *BglIII/XbaI* of the Luciferase (Luc) gene from INSM1-Luc(9). INSM1-YCD-YUPRT.FLAG: A FLAG (DYKDDDDK) tag was added to the C-terminal of the YCD-YUPRT gene reading frame by PCR-amplification using a primer pair amplifying a 500 bp fragment of the 3' part of the coding sequence, where the FLAG tag and stop codon were included in the reverse primer. Reverse primer: 5'-GGGCATGCTTACTTATCATCATCATCTTTG-TAGTCAACACAGTAGTATCTGTACCAAA-3'; forward primer: 5'-CAAAGGGACGAGGAGACTGC-3'. The amplified fragment was cut with *AgeI* and *SphI* and inserted into INSM1-YCD-YUPRT (*AgeI/SphI*) vector.

Transient transfections

For all cell lines, 2×10^6 cells were transfected with 3 μ g plasmid using 12 μ L Lipofectamine 2000 (LF) in Opti-MEM Reduced Serum Medium (Invitrogen) for 3 hours. Adherent cells were plated 1 day prior to transfection. Transfection efficiency was estimated

by the transfection of EGFP-N1 plasmid and manual scoring of the proportion of cells expressing EGFP with fluorescence microscopy.

Cell viability assays

Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma, Broendby, Denmark) addition of 20 μ l 5 mg/ml MTT solution (dissolved in sterile water) to each well and incubation for 4 hours before addition of 100 μ l solubilization buffer (10 % SDS, 0.01 M HCl). Absorption at 570 nm was measured the following day.

Cells were replated 1 day after transfection at concentrations of $0.025\text{--}0.05 \times 10^6$ cells per well in 96 well plates and incubated for 7 days with either 100 μ l growth medium or medium containing prodrug or drug: GCV (Cymevene®, Roche, Hvidovre, Denmark 196 mM) dissolved in sterile water, 5-FC (Sigma, 50 mM) dissolved in sterile water and 5-FU (Mayne Pharma, Hellerup, Denmark, 50 mg/ml)

In bystander mixed cell assays, transfected cells were mixed with untransfected parental cells (1 day after transfection) in defined ratios, plated and incubated for 7 days.

In bystander medium assays, untransfected cells were plated with conditioned medium from suicide gene transfected/prodrug treated cells. The conditioned medium was prepared as follows: cells were transiently transfected at day 1, prodrug was added at day 2 and the growth medium harvested at day 4.

Western blot analysis

Whole cell lysates of cells were prepared by sonication in ice-cold Tris-HCl 20 mM (pH 7.5), Triton X-100 2 % supplemented with protease and phosphatase inhibitor mixture II and III (Calbiochem, Roedovre, Denmark). Protein concentrations were determined using the BCA protein assay (Pierce, Herlev, Denmark) according to manufacturers instructions. Western blot was performed using 4–12 % Bis-Tris gels, loading 20 μ g protein, in the NuPAGE PreCast Gel System (Invitrogen). *Primary antibodies*: rabbit polyclonal anti – DDDDK (FLAG) (1:4000, Abcam, Cambridge, United Kingdom), rabbit monoclonal-anti-tubulin (1:1000, Cell Signaling, MA, USA), rabbit polyclonal anti-connexin 43 (1:2000, Sigma). *Secondary antibodies*: swine anti-rabbit IgG (1:1000, DAKO, Glostrup, Denmark). Signals were detected utilizing SuperSignalRWest Dura extended Duration Substrate (Pierce) in the UVP Biospectrum®AC imaging system (AH Diagnostics, Aarhus, Denmark).

Animal experiments

The SCLC xenograft model was established by the injection of 5×10^6 NCI-H69 cells pr flank subcutaneous (s.c.) into 6–8 weeks old male Nude NMRI mice (Taconic, Aarhus, Denmark). Tumors from injected mice (termed passage 0) were used for serial transplantation of mice that entered treatment protocols (passage 1) or used for serial transplantation of new animals (passage 2). Untreated tumors from each passage were subjected to pathological analysis to evaluate the existence of clinically validated SCLC markers.

The following treatment set-up was performed in two independent studies with treatment of mice from passages 1 and 2. Approximately 2 weeks after transplantation visible tumor nodules appeared and were measured every second day with caliper to confirm exponential tumor growth before treatment start. Tumor volume was calculated using the formula: $\pi/6 \cdot (d1 \cdot d2)^{3/2}$, where d1 and d2 are diameters at perpendicular angles(26) After 3–4 weeks tumors had reached a size of 200–600 mm³ and mice with tumors undergoing sustained

growth were randomized into three treatment groups. The first two groups received either intratumoral (i.t.) injections of 100 μ l of INSM1-YCD-YUPRT.FLAG or INSM1-LUC (mock) vector encapsulated in DOTAP/Cholesterol liposome (see below), while the third group received i.t. injection of 100 μ l 5 % glucose (D5W (Sigma)) used for DOTAP/Cholesterol:DNA mixing. From day 1 of i.t. injections 500 mg/kg of 5-FC (Ancotil, Meda AS, Allerød) were injected intraperitoneally (i.p.) daily for 10 days. When tumors reached 1000 mm³, the mice were sacrificed and tumor tissue and major organs were resected. For detection of transgene expression in tumor tissue, mice were treated 3 consecutive days with DOTAP/Chol encapsulated INSM1-YCD-YUPRT.FLAG or EGFP-N1, sacrificed at day 4 and tumor tissue resected. All animal experiments were performed in accordance with ethical guidelines under valid licence from the Danish Animal Experimentation Board.

DOTAP/cholesterol:DNA lipoplex preparation for i.t. injections

The DOTAP/Cholesterol (DOTAP/Chol) reagent was prepared as follows: DOTAP (N-[1-2,3-dioleoyl]propyl]-N, N, N-trimethylammonium chloride, Avanti Polar Lipids Inc., Alabaster, AL, USA) and synthetic cholesterol (Sigma), 140 μ mol each, were mixed with rotation and dissolved in a total volume of 7 ml chloroform in a glass flask and fitted in a rota-vaporator-R (Büchi, Roland Carlberg Proces system, Göteborg, Sweden). The solvent was evaporated with rotation under a nitrogen gas stream at 30 °C. The lipid film was dried by applying high vacuum drying for 45 min. followed by hydration of the lipid film in 7 ml of D5W resulting in a 40 mM total lipid solution. The tubes were sealed and placed at 50 °C for 30–60 minutes with repeated rotary moment to ensure complete hydration of the lipids on the glass surface and left overnight at room temperature. The next day liposome preparations were sonicated in a sonication water bath (Branson ultrasonic model B.V., Danbury, CT, USA) for 5 minutes at 50 °C and then downsized using a small-scale extruder (Avanti Polar lipids Inc.) with polycarbonate nanopore filters (400 nm, 200 nm and 100 nm, Whatman, Frisenette, Knebel, Denmark) at 50 °C performing 11 passes for each filter size. Size and charge (zeta potential) of particles was assessed as described previously(29) and was found to be 100 ± 2 nm and +45 mV before mixing with DNA. After mixing, lipoplex size was typically 450 ± 2 nm and +40 mV. For mixing with DNA, 20 μ l of DOTAP/Chol was diluted in D5W and mixed with DNA solutions containing INSM1-YCD-YUPRT.FLAG or INSM1-LUC (45 μ g) respectively, by rapid pipetting up and down, yielding a total volume of 100 μ l DOTAP/Chol:DNA lipoplex solution. Within 1–2 hours of mixing the lipoplexes were injected i.t. into xenografted tumors.

Tissue preparation and analysis

Mice tissues were either fixed overnight in freshly prepared neutral 4% paraformaldehyde (PFA) followed by incubation in 70 % ethanol or freshly frozen in O.M.T. Tissue-Tech (Sakura Finetek, Vaerlose, Denmark) and liquid nitrogen. PFA fixed tissue were embedded in paraffin and cut to 4 μ m sections in a routine fashion on plus coated slides. The slides were deparaffinized, hydrated and stained with hematoxylin and eosin or using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to manufacturers instructions except for previously described modifications(30;31). Polyclonal rabbit anti-GFP (1.3500, Abcam) antibody was used to detect EGFP expression and polyclonal rabbit anti-FLAG (DDDDK) (1:20000, Abcam) was used to detect YCD-YUPRT.FLAG expression. Sections were counterstained with hematoxylin and mounted for microscope evaluations using an Olympus BX51 microscope (Olympus A/S, Ballerup, Denmark). Frozen tissue sections (6 μ m) were prepared using a cryostat microtome in a routine fashion. After thawing, sections were mounted and immediately analyzed by Olympus BX2 confocal laser scanning microscopy (emmission 488 nm, excitation 530 nm) using Fluoview software version 2.1 (Olympus).

Software and statistics

Data from *in vitro* cytotoxicity assays were obtained from at least 3 independent experiments each performed in triplicates or quadruplicates. *In vivo* data were obtained from two independent studies. For all experiments data were plotted as mean $\pm$ standard error of the mean (SEM) unless otherwise stated and non-linear regression analysis was performed using GraphPad Prism™ 3.0 software (Inno-Max, Aabybro, Denmark). All mean comparisons are based on the Student's t-test at indicated significance level.

Results

INSM1 promoter driven YCD-YUPRT/5-FC suicide gene therapy induces high and specific cytotoxicity superior to YCD/5-FC in SCLC cell lines

The three SCLC cell lines GLC16, NCI-H69 and DMS53, which were established at different laboratories and exhibit different growth characteristics(32–34), were selected to obtain a SCLC cell culture model for the evaluation of INSM1-driven suicide gene therapy. We have previously tested INSM1 promoter activity in a large number of SCLC cell lines showing that the promoter exhibit 2–10 fold higher activity than the constitutive active SV40 promoter. Among the number of cell lines tested, DMS53 showed relative low activity from the INSM1 promoter while NCI-H69 and GLC16 exhibited medium to high INSM1 promoter activity(8;9) Different levels of INSM1-driven suicide gene expression could therefore be expected in these cell lines. Furthermore, transgene transfection efficiency varies between the cell lines with $60 \pm 3\%$ efficiency in GLC16, $45 \pm 2\%$ in NCI-H69 and $25 \pm 3\%$ in DMS53.

To evaluate the effect of CD-based suicide gene therapy on SCLC cell viability, INSM1-YCD, INSM1-YCD-YUPRT and pGL3 basic (mock) transfected cells were treated with increasing concentrations of the prodrug 5-FC. Cell cytotoxicity from treatment was measured and inhibitory concentration causing 50 % reduction in cell viability (IC50) was calculated (Figure 1A). Significant cytotoxicity was observed in all cell lines after INSM1-YCD/5-FC or INSM1-YCD-YUPRT/5-FC treatment. Importantly, no cell death was observed in mock-transfected cells upon exposure to 5-FC. Highest sensitivity was in general obtained in GLC16 (1000 μ M 5-FC, $p < 0.0005$) followed by NCI-H69 (1000 μ M 5-FC $p < 0.005$) and last DMS53 (1000 μ M 5-FC $p < 0.01$) and in all cell lines the YCD-YUPRT fusion gene rendered cells significantly more sensitive to prodrug treatment than the YCD gene alone. To investigate whether variations in the sensitivity towards 5-FU influenced the cytotoxic effect of the suicide gene therapy in the cell lines, untransfected GLC16, NCI-H69 and DMS53 cells were exposed to increasing 5-FU concentrations. No significant difference in IC50 values was observed (data not shown) excluding any differential 5-FU sensitivity to influence gene therapy response rates.

To clarify transgene product levels in the cells, the YCD-YUPRT fusion gene was tagged C-terminally with the FLAG (DYKDDDDK) epitope. Following transfection with INSM1-YCD-YUPRT.FLAG or mock vector, Western Blot analysis was performed on total protein lysates of transfected cells (Figure 1B). In agreement with cytotoxicity data (Figure 1A), the highest level of transgene expression was detected in GLC16 followed by NCI-H69, while only trace levels were detectable in DMS53 cells.

To explore the ability of the YUPRT part of the YCD-YUPRT fusion gene to increase 5-FU toxicity, mock-INSM1-YCD and INSM1-YCD-YUPRT transfected cells were exposed to 5-FU and cell viability evaluated. In NCI-H69 and DMS53 cells, 5-FU treatment was equally toxic to both mock, YCD and YCD-YUPRT-transfected cells (data not shown). However, in YCD-YUPRT transfected GLC16 cells, significantly higher cytotoxicity ($p < 0.05$) was observed upon 5-FU exposure compared to YCD and mock-transfected cells (Figure 1C).

This demonstrates the capability of the transfected YUPRT transgene to enhance 5-FU toxicity in this cell line. Although the specificity of the INSM1 promoter previously has been thoroughly investigated(9;10), the strong effects of YCD-YUPRT/5-FC therapy in SCLC prompted us to confirm that no trace transgene expression in cells of other origin than SCLC would allow for cytotoxicity with the potent YCD-YUPRT/5-FC system. For that purpose, the non-SCLC cell line H1299 and the glioblastoma multiforme cell line U87MG were transiently transfected with INSM1-YCD-YUPRT vector and exposed to increasing concentrations of 5-FC and 5-FU (Figure 1D). Both cell lines exhibit transfection efficiencies (60–80%, data not shown) comparably higher than the three SCLC cell lines. Both suicide gene- and mock-transfected cells were highly sensitive to 5-FU treatment ($p<0.01$) confirming sensitivity to the toxic end-product. However, no cytotoxicity was observed from INSM1-YCD-YUPRT/5-FC therapy manifesting the high specificity of the gene therapeutic strategy.

INSM1-YCD-YUPRT/5-FC therapy is superior to INSM1-HSVTK/GCV in SCLC cell lines

In order to assess whether INSM1-YCD-YUPRT/5-FC therapy is superior to INSM1-HSVTK/GCV therapy a direct comparison of the systems was performed in GLC16, NCI-H69 and DMS53 cells (Figure 2A). For YCD-YUPRT therapy the maximum tolerated dose of 5-FC is 1000 μM (Figure 1A), while a maximum tolerated dose of 10 μM GCV was established for HSVTK therapy (data not shown). YCD-YUPRT/5-FC therapy induced significantly higher cytotoxicity in all tested SCLC cell lines compared to HSVTK/GCV therapy ($p<0.05$ or $p<0.005$ as indicated). Interestingly, with INSM1-HSVTK/GCV therapy the highest efficacy was obtained in NCI-H69 followed by GLC16, while no cytotoxicity could be detected in the DMS53 cell line. To improve HSVTK-based treatment another prodrug analogue, penciclovir (PCV) was tested. PCV has been suggested as a more suitable prodrug candidate for HSVTK suicide gene therapy due to lower intrinsic activity and a pronounced safer clinical profile(19;35). PCV could be applied at a dose up to 100 μM without affecting mock-transfected cells (results not shown), but did at this concentration cause significantly less cytotoxicity than GCV ($p<0.05$) in HSVTK-transfected GLC16 and NCI-H69 cells (Figure 2A). Clearly, no advantage from replacing GCV with PCV could be obtained.

We went on to investigate whether cytotoxic bystander effects from HSVTK/GCV treatment was present in treated SCLC cells. When cultured medium from INSM1-HSVTK/GCV treated NCI-H69 or GLC16 cells was transferred to untransfected parental cells, no reduction in cell viability could be observed in accordance with the notion that phosphorylated GCV compounds are trapped in the producer cells(6) (data not shown). To further investigate GJIC-dependent bystander effects, we investigated the gap junctional status in the cell lines. Gap junctions are made up of connexin proteins in which connexin 43 is the most widely expressed subform(36) and therefore also an established marker for functional GJIC(17). We therefore investigated, connexin 43 levels by Western Blot analysis performed on total protein lysates from the cell lines and two diploid non-immortalized lung fibroblast cell lines CCD32Lu and CCD19Lu (Figure 2B). The lung fibroblasts were included to represent normal healthy lung cells, which is known to express significant levels of connexin 43(37) as also demonstrated in the Western Blot analysis. Of the cancer cells, only the NCI-H69 cell line expressed detectable levels of connexin 43 correlating with the fact that highest effect of HSVTK/GCV treatment is obtained in this cell line (Figure 2A).

INSM1-YCD-YUPRT/5-FC therapy causes a pronounced bystander effect in SCLC cells

In contrast to phosphorylated GCV compounds 5-FU and downstream toxins are not trapped in the cell and are able to diffuse freely over the cellular membrane. To explore the bystander effect of INSM1-YCD-YUPRT/5-FC gene therapy, cultured medium from treated

GLC16, NCI-H69 and DMS53 cells was transferred to untransfected parental cells. Cell viability data demonstrated that cultured medium caused massive cell death to untreated cells (Figure 3A). To further quantify the bystander effect, INSM1-YCD-YUPRT-transfected cells were mixed with untransfected parental cells in different ratios and exposed to 5-FC (Figure 3B). Significant bystander cytotoxicity was observed in all cell lines with the most pronounced effects in GLC16 and NCI-H69. Bystander cytotoxicity increased with increasing 5-FC doses rendering 70–100 % of GLC16 cells and 30–80% of NCI-H69 cells at all mixing ratios sensitive to treatment with 500 μ M 5-FC. More modest bystander effects was observed in DMS53, however, when mixing transfected GLC16 cells with untransfected DMS53 at different ratios, a similar reduction of cell viability was observed in this cell line (Figure 3C).

No further cytotoxicity is obtained from combining INSM1 promoter driven HSVTK(GCV and YCD-YUPRT/5-FC therapy in SCLC cells

Although INSM1-HSVTK/GCV therapy clearly is less effective than INSM1-YCD-YUPRT/5-FC therapy in SCLC cell lines we investigated the combined therapeutic effect of the systems, since others previously have shown synergistic interactions between HSVTK/GCV and CD/5-FC therapy(38;39). For that purpose NCI-H69 cells were transfected with equal amounts of INSM1-HSVTK and INSM1-YCD-YUPRT vectors followed by concomitant exposure to 5-FC and GCV (Figure 4). Using a fixed GCV concentration at 10 μ M (maximum tolerated) it was observed that significant enhancement of cytotoxicity was achieved at 5-FC doses at or lower than 100 μ M ($p < 0.05$). However, no additional cytotoxicity from HSVTK/GCV therapy was obtained by applying higher 5-FC doses. Hence, the advantage from combining HSVTK/GCV and YCD-YUPRT/5-FC treatments was neutralized at optimal 5-FC doses.

INSM1-YCD-YUPRT/5-FC treatment of SCLC xenografts induces significant tumor growth delay

Having observed specific and high therapeutic activity of INSM1-YCD-YUPRT/5-FC therapy *in vitro* we proceeded to evaluate the system *in vivo* on SCLC xenografted tumors. For gene delivery, the liposome based nanoparticle DOTAP/Cholesterol (DOTAP/Chol), which is an established non-viral delivery system in clinical trials for the treatment of non-SCLC(28;40;41), was used. For xenograft model we chose to use the NCI-H69 cell line, since this cell line shows much better growth and propagation properties than GLC16 and DMS53 (data not shown) and hence, represents a better model of aggressively growing SCLC. NCI-H69 cells were xenotransplanted on nude mice and treatment initiated when tumor size had reached 200–600 mm³ (Figure 5A insert) to allow for sustained and very aggressive tumor growth (data not shown) at the initiation of therapy.

Tumor-bearing mice were randomized into three treatment groups of which the first two groups received DOTAP/Chol encapsulated INSM1-YCD-YUPRT.FLAG or INSM1-LUC (mock control) vector injected intratumorally (i.t.) once daily for 3 consecutive days. The third group was injected i.t. with D5W. From day 1 of treatment, 500 mg/kg of 5-FC was administered once daily for 10 days to all groups and tumor size measured daily by caliper. As shown in Figure 5A significant tumor growth delay of the INSM1-YCD-YUPRT/5-FC treated mice was observed ($p < 0.001$) compared to mock- and D5W-treated mice as reflected by a significant increase in tumor doubling time (T_d) (figure 5A, table). No systemic toxicity was induced from treatments as evaluated by histology of resected major mice organs and mice weight data (results not shown). Tumor growth restraint of the mock-treated tumors was observed compared to the D5W-treated tumors presumably due to an unspecific anti-tumor effect of lipoplexes as previously reported by others(41–43). Also, mice treated i.t with. DOTAP/Chol:INSM1-YCD-YUPRT without administration of 5-FC showed similar

tumor growth delay to DOTAP/Chol:INSM1-LUC/5-FC treated mice (data not shown) emphasizing that the unspecific effect was indeed caused by the lipoplexes and not due to an unspecific effect of the mock plasmid. After 8 days, all (7/7) D5W-treated mice were withdrawn from treatment due to maximal tumor sizes (1000 mm³) while 3/11 (27%) mock-treated mice and 6/12 (50%) suicide gene-treated mice survived more than 10 days. To relate *in vivo* efficacy to transfection efficacy of DOTAP/Chol:DNA lipoplexes of SCLC tumors, EGFP-N1 or INSM1-YCD-YUPRT.FLAG vectors were encapsulated in DOTAP/Chol and injected i.t. for 3 consecutive days and tumor tissue resected at day 4 for immunohistochemical and fluorescence microscopy analysis of EGFP and YCD-YUPRT (FLAG) expression (Figure 5B). Distinct areas of EGFP and FLAG expression was found distributed throughout the tumor confirming the transfection capability of the lipoplex formulation.

Discussion

In this study we demonstrated that INSM1-driven(8;9) YCD-YUPRT/5-FC therapy induces significant cytotoxicity in SCLC cell lines superior to YCD/5-FC (Figure 1B) and HSVTK/GCV (Figure 2A), which until the present study was the only suicide gene strategy tested for SCLC(9–16). Importantly, the INSM1-YCD-YUPRT/5-FC strategy was also investigated *in vivo* demonstrating significant tumor growth delay compared to control treatment. This is the first study to describe INSM1-driven YCD-YUPRT/5-FC therapy and the first to investigate the therapeutic potential of delivery of suicide gene therapeutics for SCLC *in vivo*. In the only previous *in vivo* study investigating gene therapy for SCLC, cancer cells were adenovirally transduced *ex vivo* before the injection on mice for xenograft establishment(15).

Since INSM1 promoter activity is increased in all tumors of neuroendocrine origin it is of interest to test the promoter in suicide gene therapy for other neuroendocrine tumors than SCLC. In one very recent study the potential of the INSM1 promoter for regulated HSVTK/GCV therapy for primitive neuroectodermal tumors was demonstrated *in vitro* and *in vivo*(44).

The majority of studies describing the CD-UPRT fusion strategy, have utilized the *E. coli* ortholog of CD-UPRT(25–27;45) although the superiority of the yeast CD compared to the *E. Coli* version previously has been established(21–23). Additionally, only very few studies have focused on the regulated expression of CD-UPRT from cancer-relevant regulatory elements. By far the majority of studies have demonstrated CD-UPRT/5-FC efficacy driven from the constitutive active CMV promoter(23–27;45) even though this strategy is not feasible for systemic treatment due to the constitutive activity of the CMV promoter in normal tissues. The few attempts to include cancer-regulated expression is represented by a study of paclitaxel-resistant ovarian cancer(45) and a study of prostate cancer using the MDRI and prostate specific membrane antigen (PSMA) promoter(46), respectively, for the regulated expression of the *E. Coli* CD-UPRT. However, both the MDR1 and PSMA gene is expressed in a range of normal healthy tissues(47;48), which highly questions the safety of exploiting these promoters for suicide gene therapy.

In the present study we show that INSM1-regulated YCD-YUPRT/5-FC therapy is convincingly more effective than HSVTK/GCV therapy in SCLC cell lines (Figure 2A). In an attempt to improve HSVTK/GCV effect, we replaced GCV with the prodrug PCV, which has been suggested to be a more suitable prodrug candidate for HSVTK therapy. Although PCV could be applied in higher doses than GCV without affecting mock-treated cells (data not shown), the prodrug induced significantly less cytotoxicity to HSVTK-positive cells compared to GCV (Figure 2A). The compromised effect of HSVTK/GCV treatment

prompted us to investigate if GJIC-dependent bystander effects were compromised in the SCLC cells. Western blot analysis, failed to detect expression of the GJIC marker, connexin 43 in GLC16 and DMS53 cells, while detectable levels of the marker was observed in NCI-H69 (Figure 2B). The expression of connexin 43 in NCI-H69 correlated with the fact that this cell line showed highest sensitivity to HSVTK/GCV treatment of the three cell lines. To further investigate the expression of connexin 43 and GJIC in SCLC, we have tested a large number of SCLC cell lines and xenografts for connexin 43 expression (results not shown). Overall no difference in connexin 43 levels was observed between cell lines and established xenografts excluding any *in vivo* related events to influence connexin 43 expression. Only few of the SCLC cell lines and corresponding xenografts had detectable connexin 43 and in these, as for NCI-H69, expression was subtle compared to the diploid lung fibroblast cell lines CCD32Lu and CCD19Lu. The results together indicate that the potential of HSVTK/GCV therapy may be limited in SCLC due to compromised GJIC-dependent bystander effect in this malignancy.

Since others have shown a synergistic interaction between CD/5-FC and HSVTK/GCV suicide gene therapy(38;39) we explored the possibility to combine HSVTK/GCV and YCD-YUPRT/5-FC therapy to achieve increased therapeutic effect. Since the NCI-H69 cells showed highest sensitivity towards HSVTK/GCV treatment we initially explored the combined effect in this cell line. However, no advantage from combining systems was achieved at optimal 5-FC doses (Figure 4). The combination of HSVTK and YCD-YUPRT fusion gene has not previously been tested but clearly the potent effect of YCD-YUPRT therapy does not benefit from this combination. However, the combination of YCD-YUPRT with other potential suicide gene systems should be investigated to explore synergistic possibilities.

The individual response rates in the cell lines upon INSM1-YCD-YUPRT/5-FC therapy, was found to correlate to transgene expression levels (Figure 1A and B). In GLC16, all cells died from the administered suicide gene therapy. Additionally, increased sensitivity towards 5-FU in YCD-YUPRT-transfected cells was observed in GLC16 (Figure 1C) but not in NCI-H69 and DMS53 (not shown), presumably due to the higher levels of YCD-YUPRT in this cell line. Importantly, even though only very low transgene expression levels could be detected in DMS53, more than 50 % of cells succumbed to YCD-YUPRT/5-FC therapy in this cell line (Figure 1B). Since DMS53 was equally sensitive to 5-FU treatment as GLC16 and NCI-H69 (data not shown) the lack of response in this cell line was directly correlated to low transgene expression level and not related to treatment resistance. Furthermore, we showed that bystander effects from treated GLC16 cells effectively targeted DMS53 cells demonstrating that low-transgene expressing cells can be targeted by exposure to high doses of bystander toxins from high-transgene expressing cells (Figure 3A, B and C). Taken together these data show that highly effective therapy can be obtained with INSM1-driven YCD-YUPRT/5-FC therapy in SCLC. Importantly, effective therapy can be obtained in spite of putative variations in the transcriptional activity from the INSM1 promoter, which could be expected to exist in a heterogeneous cancer cell population.

The clinical relevance of the strategy was confirmed *in vivo* (Figure 5A). Despite of very aggressive tumor growth, DOTAP/Chol:INSM1-YCD-YUPRT/5-FC treatment caused significant tumor growth attenuation compared to control treated animals, demonstrating proof-of-concept that a specific anti-tumor effect can be obtained by delivery of suicide gene therapeutics to SCLC *in vivo*. The DOTAP/Chol:DNA lipoplexes was shown to convey small foci of transgene expression throughout the tumor (Figure 5B). However, the distinct areas and low staining intensity of transgene expression in the tumor tissue as especially demonstrated from FLAG detection as shown in Figure 5B from INSM1-YCD-YUPRT.FLAG treatment, strongly indicate that higher treatment effect would be obtained

be increasing delivery efficiency and thereby transgene expression. Significant anti-tumor effects of DOTAP/Chol-mediated gene delivery has been demonstrated in non-SCLC preclinical models(40;41;43) and patients(28), however, no other studies than the present has tested the DOTAP/Chol formulation for SCLC and other delivery formulation might be more suitable for this malignancy.

It is likely that bystander effects influence the positive treatment response when considering the *in vitro* results (Figure 3) and the low level and spread of transgene expression (Figure 5B). However, this remains speculative and to further quantify this effect we attempted to compare suicide transgene expression with cell death using TUNEL and FLAG staining on consecutive sections of tumor tissue. Due to the aggressively growing properties of tumors and the fact that treatment was performed with direct i.t. injections of DOTAP/Chol:DNA lipoplexes causing general toxicity, resected SCLC tumors had large regions of necrosis and apoptosis as evaluated by H&E and TUNEL staining (results not shown), which hindered thorough evaluation of bystander effects. However, a quantitative bystander model could be performed by mixing stable INSM1-YCD-YUPRT.FLAG expressing cells with untransfected parental cells in different ratios for xenograft establishment. Treatment of small-size mixed xenografts with 5-FC followed by evaluation of FLAG and TUNEL staining on resected tissue might clarify the level of bystander influence on anti-tumor responses *in vivo*.

It would be highly relevant to further test the presented treatment strategy in SCLC preclinical models which mimic the metastatic nature of SCLC and hereby develop systemic treatment formulations and schedules which could be adapted into the clinic. Orthotopic disseminated models have successfully been developed for non-SCLC(40;41;43) and treatment of DOTAP/Chol based gene therapy in such models have resulted in dramatic anti-tumor responses due to the distribution of the DOTAP/Chol lipoplexes in particular to intrathoracic organ sites. Kuo *et al.* (49) has demonstrated seeding of SCLC cells in lung, heart and liver after intravenous injection of SCLC cells. However, in a later study of Moreira *et al.*(50) no seeding of SCLC cells could be detected in lung or other organs even after several attempts of orthotopic reconstitution and to our knowledge no other studies have been performed with orthotopic SCLC models. Thus, further clarification of the establishment of orthotopic disseminated models for SCLC is needed.

The results in the present study yield promise for the future development of YCD-YUPRT/5-FC suicide gene therapy towards the design of a clinical protocol for the treatment of SCLC.

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Translational Relevance

Since current treatment protocols for the management of small cell lung cancer (SCLC) are inefficient the advancement of novel therapies should have high priority. In transcriptionally targeted suicide gene therapy a cancer-specific promoter regulates the expression of a suicide gene, which promotes the efficient conversion of a non-toxic prodrug into a cell toxin. In the present study we evaluate the effect of the suicide gene yeast cytosine deaminase fused to uracil phosphoribosyltransferase under transcriptional control of the SCLC specific INSM1 promoter. The strategy induces massive and specific cell death in SCLC cells lines and significant tumor growth delay upon *in vivo* delivery of suicide transgene using DOTAP/Cholesterol liposome, an established delivery vehicle for lung cancer. The study demonstrates that highly effective and specific therapy can be obtained in SCLC using transcriptionally targeted suicide gene therapy and suggests that initiative should be taken to implement this strategy to replace or supplement existing therapies for SCLC

Figure 1. Effect of INSM1-driven YCD/5-FC and YCD-YUPRT/5-FC therapy in SCLC cell lines and cell lines of other origin than SCLC

A) Cytotoxicity assays of the SCLC cell lines GLC16, NCI-H69 and DMS53 transiently transfected with pGL3 basic (mock), INSM1-YCD or INSM1-YCD-YUPRT vectors followed by exposure to 0–1000 μ M 5-FC. Below graphs IC₅₀ values in μ M are shown as mean (95 % confidence interval), calculated by non-linear regression analysis. B) Detection of YCD-YUPRT.FLAG expression in GLC16, NCI-H69 and DMS53 after transient transfection with either pGL3 basic (mock) or INSM1-YCD-YUPRT.FLAG vector. Cells were harvested and total protein lysates were subjected to Western Blot analysis with anti-FLAG antibody. Anti-Tubulin staining serves as loading control. C) Cytotoxicity assay of GLC16 transiently transfected with pGL3 basic (mock), INSM1-YCD and INSM1-YCD-YUPRT and exposed to 1 μ M of 5-FU. * represents significant cytotoxicity ($p < 0.05$) of INSM1-YCD-YUPRT/5-FU therapy compared to mock/5-FU and INSM1-YCD/5-FU. D) Cytotoxicity assay of the non-SCLC cell line H299 and the glioblastoma cell line U87 MG transiently transfected with pGL3 basic (mock) or INSM1-YCD-YUPRT and exposed to 1000 μ M 5-FC or 1 μ M 5-FU as indicated. ** represents significant cytotoxicity ($p < 0.01$) of mock/5-FU treated cells compared to mock/5-FC treated cells. In both A), C) and D) cytotoxic effects were measured by an MTT assay. Data are normalized to control treated cells (no exposure to 5-FC or 5-FU) set to 100 % cell viability for each treated cell population.

Figure 2. Comparison of cytotoxic effect of INSM1-driven YCD-YUPRT/5-FC, HSVTK/GCV and HSVTK/PCV therapy and connexin 43 status in SCLC cell lines

A) Cytotoxicity assays of the SCLC cell lines GLC16, NCI-H69 and DMS53 transiently transfected with INSM1-YCD-YUPRT or INSM1-HSVTK vectors followed by exposure to 1000 μ M 5-FC for YCD-YUPRT-transfected cells and 10 μ M GCV or 100 μ M PCV for HSVTK-transfected cells. * represents significant difference in cytotoxicity at $p < 0.05$ and ** at $p < 0.005$. Cytotoxic effects were measured by an MTT assay. Data are normalized to control treated cells (no exposure to 5-FC, GCV or PCV) set to 100 % cell viability for each treated cell population. HSVTK/PCV treatment was not performed in DMS53 (n.d.) B) Detection of connexin 43 expression in GLC16, NCI-H69 and DMS53 and the diploid lung fibroblasts CCD19Lu and CCD32Lu. Cells were harvested and total protein lysates were subjected to Western Blot analysis with anti-connexin 43 antibody. Anti-Tubulin staining serves as loading control.

Figure 3. Bystander cytotoxicity of INSM1-YCD-YUPRT/5-FC therapy in SCLC cell lines

A) Untransfected cells exposed to conditioned medium from mock- or INSM1-YCD-YUPRT-transfected cells exposed to 5-FC for 2 days. * represents significant difference in cytotoxicity at $p < 0.05$ and *** at $p < 0.001$ compared to mock-transfected cells. B) INSM1-YCD-YUPRT-transfected cells (T) were mixed with untransfected parental cells (UT) in indicated T:UT ratios followed by exposure to 5-FC. C) INSM1-YCD-YUPRT-transfected GLC16 cells (T) were mixed with untransfected DMS53 cells (UT) in indicated T:UT ratios followed by exposure to 5-FC. In B) and C) * represents significant difference in cytotoxicity at $p < 0.05$, ** at $p < 0.005$ and *** at $p < 0.001$ compared to untransfected cells (UT) alone. In A), B) and C) cytotoxic effects were measured by an MTT assay. Data are normalized to control (exposure to 0 μ M 5-FC) set to 100 % cell viability for each treated cell population

Figure 4. Effect of combined suicide gene therapy with INSM1-HSVTK/GCV and INSM1-YCD-YUPRT/5-FC in SCLC cells

Cytotoxicity assay of the SCLC cell line NCI-H69, transiently transfected with both INSM1-HSVTK and INSM1-YCD-YUPRT followed by exposure to 0 or 10 μ M GCV and increasing doses of 5-FC. Cytotoxic effects were measured by MTT assay. Data are normalized to control (no exposure to 5-FC or GCV) set to 100 % cell viability for each treated cell population. * represents significant cytotoxicity at $p < 0.05$ of combination suicide gene therapy (10 μ M GCV) compared to YCD-YUPRT/5-FC therapy alone (0 μ M GCV).

Figure 5. Effect of DOTAP/Chol:INSM1-YCD-YUPRT/5-FC treatment on growth of SCLC xenografts on nude mice

A) NCI-H69 tumor transplanted nude NMRI mice were treated i.t. either with DOTAP/Chol encapsulated INSM1-YCD-YUPRT.FLAG or INSM1-LUC or with only D5W for 3 consecutive days. n equals number of tumors treated. From the first day of treatment 500 mg/kg of 5-FC was administered i.p. for 10 days. Tumor volumes at treatment start are shown in insert. Start tumor volumes were set to index 1 for normalization of subsequent tumor measurements. Table below graph summarize Tumor doubling time (T_d) in days as mean (95 % confidence interval) calculated from non-linear regression analysis and number of surviving mice at day 10. Notice that there are less mice than tumors since animals were xenotransplanted on both flanks and, therefore, depending on tumor take and growth, had either one or two tumors which entered treatment. B) Resected NCI-H69 tumor untreated (-

i.t. injection) or treated 3 consecutive days with DOTAP/Chol encapsulated EGFP-N1 or INSM1-YCD-YUPRT.FLAG (+ i.t. injection) were fixed and stained for EGFP and FLAG expression, respectively. EGFP expression was furthermore directly detected by fluorescence microscopy.