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Enriching suicide gene bearing tumor cells in vivo for an increased bystander effect: a novel strategy for cancer gene therapy

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Abbreviations

AP	Alkaline Phosphatase
BE	Bystander Effect
b.i.d	bis in die (twice a day)
BSA	Bovine Serum Albumin
CD	Cytosine Deaminase
CD/UPRT	Cytosine Deaminase / Uracil Phosphorybosyltransferase
CMV	Cytomegalovirus
d	days
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxynucleotide Acid
ds	double stranded
E. coli	Escherichia coli
EDTA	Ethylenediamintetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
5-FC	5-fluorocytosine
FCU1	Cytosine Deaminase / Uracil Phosphoribosyltransferase Fusion Gene
FCS	Fetal Calf Serum
5-FU	5-fluorouracil
5-FUMP	5-fluorouridine 5'- monophosphate
5-FUR	5-fluorouridine
g	gravitational force
GD_2	Disialoganglioside 2
h	hours
HSV	Herpes Simplex Virus
i.e.	id est (that is)
i.p.	intraperitoneal
IRES	Internal Ribosomal Entry Site

i.v.	intravenous
(m)M	(milli)Mol / liter
MCS	Multiple Cloning Site
min	minutes
MTT	3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide
MW	Molecular Weight
n	number (for example number of animals, samples, etc.)
nm	nanometer
OD	Optical Density
O/N	overnight
p53	protein 53
PALA	N-(phosphonacetyl)-L-aspartate
PBS	Phosphate-buffered Saline
RNA	Ribonucleic Acid
RT	Room Temperature
s.c.	subcutaneous
U	Unit
UMP	Uridine 5´-monophosphate
VSV	Vesicular Stomatitis Virus
w/o	without
w/w	weight to weight

1. Introduction

Gene therapy is based on the idea of utilizing nucleic acids that encode proteins with a therapeutic potential. Gene therapy promises specific and efficient treatment for various diseases including inherited genetic disorders, hematological [1, 8, 20, 43, 44, 45, 55] neurological [7, 10] and cardiovascular [24, 64] diseases as well as cancer [9, 11, 27, 29, 41, 49].

The first phase I clinical trial was conducted in 1990 for the treatment of adenosine deaminase deficiency [2], an inherited genetic disease and the same year also marked the start of the first clinical trial for cancer gene therapy [53].

However, results of clinical trials have shown that, amongst other challenges, poor in vivo gene transfer efficiency poses a major hurdle for cancer gene therapy [16, 31, 46, 47, 48, 49, 65]. Thus, intense efforts are being directed at modifying vectors and delivery systems in order to improve gene transfer.

Retroviruses are well suited vehicles for gene transfer in cancer gene therapy due to their ability to only infect dividing cells and sparing post-mitotic tissues [35]. However, only a small fraction of tumor cells pass through mitosis at a given time point and retroviruses are rapidly inactivated by complement. This explains in part the still low transduction efficiency of tumor cells in vivo. Another disadvantage of retroviruses is the random viral integration in the host genome that can induce insertional mutagenesis, potentially leading to transcriptional activation of cellular genes, including oncogenes [21, 33].

Other viral vectors, like adenoviruses, posses the capability to infect also non-dividing cells which can lead to unwanted side effects when used as vectors for cancer gene therapy. Nowadays, there is a trend towards selectively-replicating adenoviruses [27] that promise high specificity for cancer cells. Nevertheless, adenoviruses can elicit severe immune responses in the host, potentially leading to a fatal outcome in patients [50].

Non viral gene transfer, e.g. via liposomes, principally is feasible in vivo. However, the moderate specificity of targeting and the lower transfection efficiencies compared to viral gene transfer make these vectors less favorable for in vivo gene transfer.

Cancer gene therapy aims to selectively kill or inhibit the growth of tumor cells, that are (or have become) resistant to conventional approaches, by various mechanisms: e.g. restoration of non-functional tumor suppressor genes

like the protein p53 [13, 57], oncogene antagonism, induction of immune response against cancer cells (e.g. via enhanced antigenicity by expression of cytokines) [54, 58, 62] or introduction of suicide genes (reviewed in [5]).

The principle of suicide gene therapy (also called gene-directed-enzyme-prodrug therapy) is to deliver a gene to tumor cells that encodes an enzyme that is not toxic per se, but able to convert a non-toxic prodrug into a potent cytotoxin. The suicide gene´s product should be different from any endogenous enzyme, so that conversion of the prodrug is exclusively possible in target cells. Beside the tumor selectivity another advantage over conventional chemotherapy are the very high concentrations of the drugs inside the tumor, unobtainable with conventional chemotherapy. Furthermore, the generation of the cytotoxin directly inside the tumor will lead to fewer unwanted systemic side effects.

One of the first approaches for negative selection (suicide gene therapy) of tumor cells was suggested by Moolten in the late 1980s: Gene transfer of the viral enzyme thymidine kinase rendered tumor cells sensitive to the anti-metabolite prodrug ganciclovir [36, 37, 38, 39].

One prerequisite for the success of this suicide gene therapy is the bystander effect, since not all of the targeted tumor cells will express the transgene. Bystander effect (BE) means killing of neighboring tumor cells that do not express the foreign enzyme. In other words: even if only a small share of the tumor cells are genetically modified and express the therapeutic gene, tumor eradication is possible [14].

The BE is mainly only due to transport via gap junctions or diffusion of cytotoxic products to neighboring cells. But it also can be induced by apoptotic factors released from dead or dying cells. In addition to that, the immune system also plays an important role in inducing killing of neighboring cells [15].

As described above, in addition to increasing gene transfer into target cells, the killing effect of therapeutic genes on surrounding non-modified bystander cells, i.e. the BE, may be increased [51]. This has been done by gene transfer or chemical upregulation of connexins to increase the number of gap junctions necessary for export of cytotoxic ganciclovir-triphosphate from cells expressing HSV thymidine kinase to bystander cells (reviewed in [34]). The BE can also be facilitated by using suicide genes that do not depend on gap junctions for a

bystander effect. The cytosine deaminase / 5-fluorocytosine (CD / 5-FC) system, for example, exerts a strong, gap junction-independent bystander effect by outward diffusion of the cytotoxic metabolite 5-fluorouracil into the extracellular space [3, 25, 26, 40].

CD is a non-mammalian enzyme that converts cytosine to uracil, but also the nontoxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil, which is known to inhibit DNA and RNA synthesis [59]. The CD / 5-FC system is a powerful extermination system, especially when CD from yeast rather than from E. coli is used [28] and has entered clinical trials [16, 17, 42]. It has been reported that tumor regression can be observed when less than 10% of the target cells express CD [26]. However, the efficiency of the BE and the share of CD expressing cells required for a complete BE is cell line dependent [6]. Considering the fact, that some tumors need a higher amount of suicide gene bearing tumor cells for a complete BE and that the transfection efficiency in clinical trials is unlikely to exceed 10% [19] other strategies have to be developed to improve gene transfer.

Providing appropriate culture conditions, CD can also function as a positive selection system in vitro [56, 63]. When de novo pyrimidine synthesis is blocked by PALA, CD-bearing cells are rescued from PALA-induced apoptosis if cytosine and also inosine are given to drive the pyrimidine salvage pathway. Positive selection hinges on the addition of inosine to metabolize sufficient uridine necessary for rescue. While CD / 5-FC constitutes a promising bifunctional suicide gene system when used with PALA, the necessity of two adjuncts (cytosine and inosine) prevents its in vivo applicability.

Here we suggest a strategy how to compensate for poor in vivo gene transfer into tumor cells by positive in vivo selection of tumor cells bearing the therapeutic gene. When a proportion of transfected cells is reached sufficient for a complete bystander effect, the killing mechanism is activated to eradicate the tumor. To make such an enrichment-eradication strategy feasible, we searched for a bifunctional suicide gene which allows for initial positive selection as well as for terminal negative selection of the suicide gene-bearing tumor cells. We show, that a fusion gene of yeast CD and uracil phosopribosyltransferase in the presence of PALA and cytosine efficiently enriches the suicide gene-bearing tumor cells in vitro and in vivo. This positive / negative selection system is depicted in Fig.1.

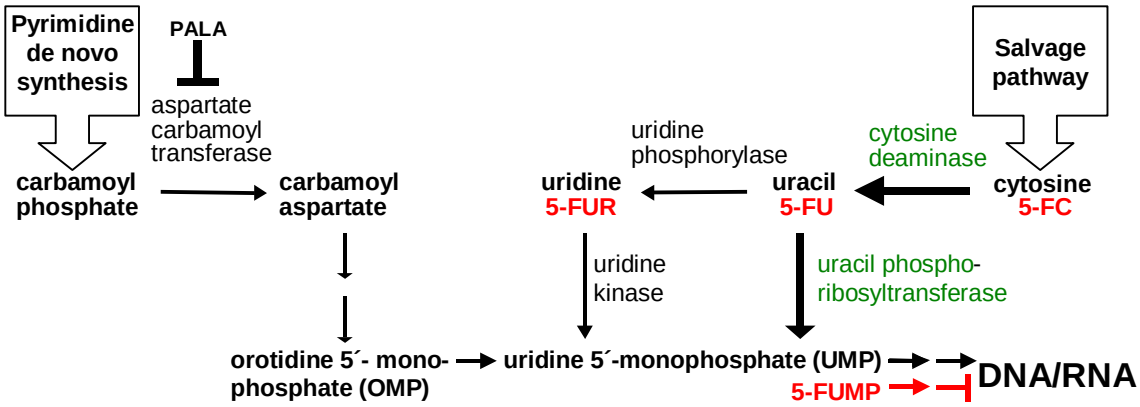

Fig.1 Pyrimidine de novo synthesis and salvage pathways: known and potential effects of FCU1, PALA, cytosine and 5-FC. Blocking aspartate carbamoyltransferase, the first enzyme of pyrimidine de novo synthesis, with the specific inhibitor PALA results in UMP depletion-induced cell death. Cells harboring the cytosine deaminase / uracil phosphoribosyltransferase fusion gene (FCU1) may overcome this pyrimidine starvation by metabolizing exogenously delivered cytosine to UMP. The uracil phosphoribosyltransferase of the fusion gene shortcuts the rate-limiting endogenous enzymes uridine phosphorylase and uridine kinase. The non-toxic prodrug 5-FC is converted by the FCU1 gene product to 5-FUMP and additional metabolites, which block DNA and RNA synthesis [59] leading to apoptosis [30].

We show that enrichment strongly augments the share of suicide gene bearing cells and therefore enhances the bystander effect in a subsequent eradication step when the prodrug 5-FC is given. This leads to a superior inhibition of tumor growth and prolonged survival in vivo compared to conventional suicide gene therapy without prior enrichment. The two-step enrichment-eradication strategy may be a promising approach to overcome low in vivo gene transfer into tumor cells, thus increasing the efficacy of cancer gene therapy.

2. Materials and methods

2.1 Materials

2.1.1 General

-80°C refrigerator (Heraeus, Hanau, Germany)
-20°C refrigerator (Liebherr, Germany)
4℃ refrigerator (Liebherr, Germany)
CC-12 camera & software (Soft Imaging System, Muenster, Germany)
CELLQuest software (Becton Dickinson, Heidelberg, Germany)
Celluloseacetate filter 0.45 µm (Carl Roth GmbH & Co, Karlsruhe, Germany)
Centrifuge (Varifuge 3.0R) (Heraeus, Hanau, Germany)
CO_2 Incubator (Heraeus, Hanau, Germany)
Digital pH Meter (210A) (Orion Research, Boston, USA)
Electrophoresis apparatus (Bio-RAD, LKB, Sweden)
Eppendorf Centrifuge 5417R (Eppendorf-Netheler-Hinz, Germany)
Ethanol (Sigma-Aldrich, Steinheim, Germany)
Excel software (Microsoft)
Flow Cytometry (FACSCalibur) (Becton Dickinson, Heidelberg, Germany)
Fluorescent microscope (AX 70) (Olympus, Stuttgart, Germany)
Hematoxylin (DakoCytomation, Glostrup, Denmark)
Inverted fluorescent microscope (Olympus, Stuttgart, Germany)
Isopropanol (Merck, Darmstadt, Germany)
Light microscope (Zeiss, Oberkochen, Germany)
Microscope slides (SuperFrost) (MENZEL, Braunschweig, Germany)
Microtome (RM 2135) (Leica Microsystems, Nussloch, Germany)
Mini centrifuge (MS Laborgeraet, Heidelberg, Germany)
MTT (Thiazolylblue) (Sigma-Aldrich, Steinheim, Germany)
Multi-channel pipette (Eppendorf, Hamburg, Germany)
Neubauer chamber (Carl Roth GmbH & Co, Karlsruhe, Germany)
Olympus digital camera (C-4040) (Olympus, Stuttgart, Germany)
Pipetter (Gilson, France)
Plate-reader (ELx 800) (Bio-Tec Instruments, Germany)

Safety Cabinet (Heraeus, Hanau, Germany)
Sterile filter (Schleicher & Schnell, Dassel, Germany)
Sterile Pipette (Becton Dickinson Labware, USA)
Sterile Syringe (Becton Dickinson Labware, USA)
Tissue Culture Flask (Becton Dickinson Labware, USA)
Tissue Culture Plate (Becton Dickinson Labware, USA)
Triton X-100 (Sigma-Aldrich, Steinheim, Germany)
UV/Visible Spectrophotometer (Phamacia Biotech, England)
Vortex (Scientific Industries, USA)

2.1.2 Cell culture

2.1.2.1 Cell lines, media, supplements

The murine neuroblastoma cell line NXS2 was obtained from R. Reisfeld at the Scripps Institute, La Jolla, CA and is syngeneic to A/J mice. NXS2 was created by fusion of disialoganglioside 2 (GD_2) negative C1300 murine neuroblastoma cells (A/J background) with murine dorsal root ganglion cells from C57Bl/6J mice, followed by fluorescence and magnetic-activated cell sorting for high GD_2 expression. GD_2 is a neuroblastoma tumor marker [32].

The packaging cell line GP293 (BD CLONTECH, Heidelberg, Germany) was used to produce pantropic retroviral particles for transduction of NXS2 cells.

The cell lines GP293, NXS2 and its derivatives (NXS2pLXIN, NXS2pL(FCU1)IN) were grown in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. This medium will be called “completed DMEM“. Fetal bovine serum was exposed to 56℃ for 30 min before adding it to the medium, in order to inactivate the complement system.

DMEM and all medium supplements were from GibcoBRL, Life Technologies, Eggenstein, Germany.

Freezing medium consisted of DMEM with 20% fetal bovine serum and 12% dimethylsulphoxide (DMSO, Sigma-Aldrich, Steinheim, Germany).

DMEM w/o Phenol Red (GibcoBRL, Life Technologies, Eggenstein, Germany)
PBS Dulbecco´s w/o Ca^{2+} / Mg^{2+} (Biochrom KG, Berlin, Germany)
Trypan Blue Solution 0.4% (Sigma Aldrich, Steinheim, Germany)
Trypsin/EDTA (Biochrom KG, Berlin, Germany)

2.1.2.2 Cell culture plastic ware

Cell culture flasks and multiwell-plates used for the experiments were BioCoat™ Collagen I tissue culture products (BD Bioscience, San Jose, CA, USA).

2.1.2.2 Drugs and chemicals

γ-cyclodextrin (Wacker Chemie, Burghausen, Germany) .

Cytosine (Sigma-Aldrich, Steinheim, Germany) was dissolved in serum-free DMEM for a 50 mM stock solution.

5-Fluorocytosine (Sigma-Aldrich, Steinheim, Germany) was dissolved in demineralized water for a 80 mM stock solution.

DL-Lactic acid (Sigma-Aldrich, Steinheim, Germany) was dissolved in demineralized water for a 1% (w/w) solution.

MTT stock solution was 5 mg MTT (Sigma-Aldrich, Steinheim, Germany) in 1 ml DMEM without phenol red (GibcoBRL, Life Technologies, Eggenstein, Germany).

MTT working solution was a 1:10 dilution of the MTT stock solution in DMEM without phenol red (GibcoBRL, Life Technologies, Eggenstein, Germany).

N-(phosphonacetyl)-L-aspartate (PALA) (NCI, Bethesda, MD, USA) was dissolved in demineralized water for a 64 mM stock solution.

2.1.3 Gene transfer

2.1.3.1 Vectors

pLXIN (BD CLONTECH, Heidelberg, Germany):

The 6.1 kb mammalian expression vector pLXIN is the backbone of pL(FCU1)IN and serves as empty vector control. It contains an internal ribosomal entry site (IRES) between the multiple cloning site and the neomycin-resistance gene. This permits both the gene of interest (cloned into the multiple cloning site) and the neomycin resistance gene to be translated from a single bicistronic mRNA. Both, the gene of interest and the neomycin resistance gene are under the control of the LTR promoter allowing stable transfected eukaryotic cells to be selected by G418. A bacterial promoter upstream of the neomycin-resistance gene expresses ampicillin resistance allowing propagation of the plasmid in E.coli.

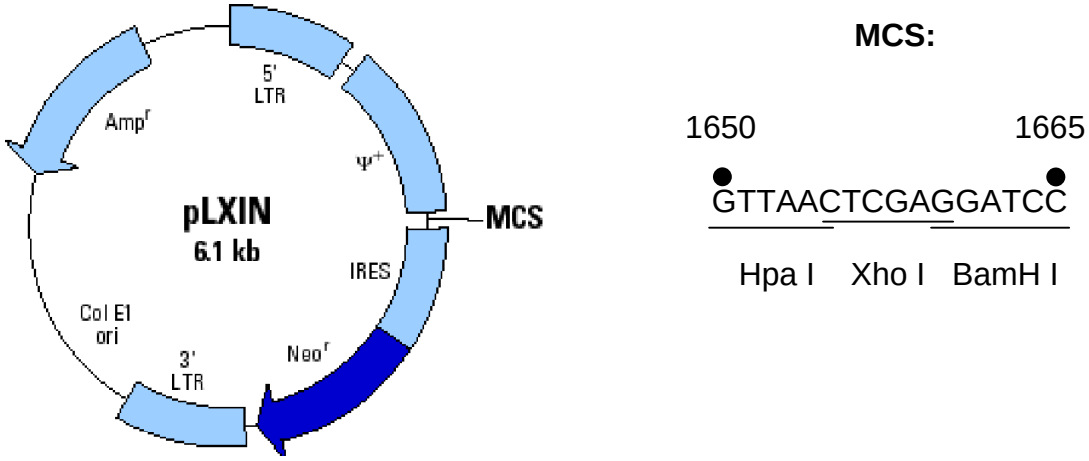

Fig.2 Retroviral vector pLXIN with Multiple Cloning Site (MCS).

pTG13046 (TRANSGENE S.A., Strasbourg, France)

The 6.6 kb mammalian expression vector pTG13046 contains the yeast (Saccharomyces cerevisiae) cytosine deaminase uracil phosphoribosyltransferase fusion gene (FCU1). The expression of the fusion gene is under the control of the CMV promoter. A neomycin resistance cassette (Neo^r) under the control of the SV40 early promoter allows stable transfected eukaryotic cells to be selected using G418. The vector also provides an ampicillin resistance gene for propagation in E.coli. FCU1 was excised from this vector and subcloned in pLXIN. The resulting vector was called pL(FCU1)IN.

pVSV-G (BD CLONTECH, Heidelberg, Germany)

The plasmid pVSV-G was used to cotransfect the packaging cell line GP-293.

This vector encodes the envelope glycoprotein from the vesicular stomatitis virus.

Thus, cotransfection with this vector results in the production of pseudotyped viral particles, that rapidly bind to the VSV receptor and enter the cell by interacting with an ubiquitous phospholipide component of the cell membrane. This means that VSV-G pseudotyped cells have a very broad tropism. Another advantage of VSV-G pseudotyped retroviral particles is that they can be concentrated due to their physical stability by ultracentrifugation to generate very high titers.

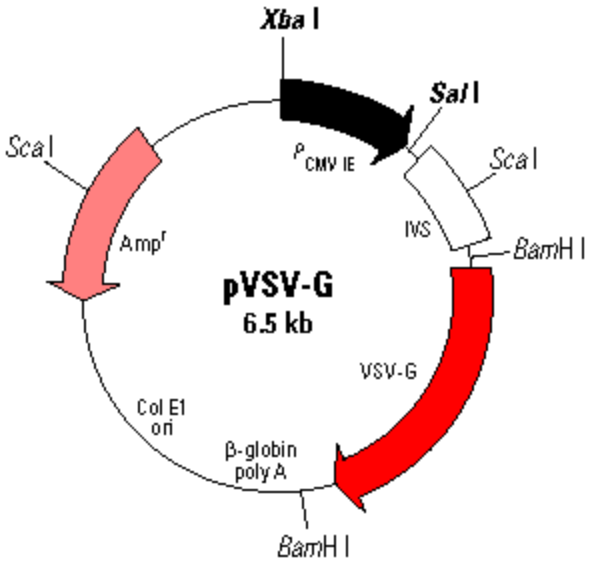

Fig.3 Plasmid pVSV-G.

2.1.3.2 Bacteria

Super competent E. coli (SCS110 strain) (Stratagene, La Jolla, CA, USA)

2.1.3.3 Reagents and kits

Agarose (Roche, Karlsruhe, Germany)
Ampicillin (Sigma-Aldrich, Steinheim, Germany)
Ethidium bromide (Sigma-Aldrich, Steinheim, Germany)
dNTP Mix (2 mM) (Roche, Karlsruhe, Germany)
GenePORTER transfection reagent (PEQLAB Biotech, Erlangen, Germany)
Geneticin (G-418 Sulphate) (Life Technologies Inc, Eggenstein, Germany)
Klenow Enzyme (Roche, Karlsruhe, Germany)
Phosphatase, alkaline, shrimp (Roche, Karlsruhe, Germany)
Plasmid maxi / mini Kit (Qiagen, Hilden, Germany)
Polybrene (Sigma-Aldrich, Steinheim, Germany)
QIAquick gel extraction Kit (Qiagen, Hilden, Germany)
QIAquick PCR purification Kit (Qiagen, Hilden, Germany)
Rapid DNA ligation Kit (Roche, Karlsruhe, Germany)
Restriction endonucleases: BamHI, SmaI, XhoI (Roche, Karlsruhe, Germany)
Trichostatin A (Sigma-Aldrich, Steinheim, Germany)

2.1.3.4 TE buffer, LB medium, LB agar

1 x TE buffer

10 mM	Tris-HCl pH 7.5
1 mM	EDTA pH 8.0

1 x LB medium

5 g/l	Sodium chloride
5 g/l	Yeast extract
10 g/l	Tryptone
10 mM	Tris-HCl pH 8.0
100 µg/ml	Ampicillin

1 x LB Agar

10 g/l	Sodium chloride
5 g/l	Yeast extract
10 g/l	Tryptone
15 g/l	Agarose
100 µg/ml	Ampicillin

2.1.4 Cell staining, Fluorescence Activated Cell Sorting (FACS)

The lipid soluble fluorescent dye 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes, MoBiTec GmbH, Göttingen, Germany.

1 x FACS buffer

5 ml	heat-inactivated fetal calf serum
500 ml	PBS without Mg^{2+} /Ca^{2+}

2.1.5 Animal experiments

2.1.5.1 Animals

6-8 week old female A/J mice were purchased from Harlan Winkelmann, Borchen, Germany. These mice are syngeneic to NXS2 neuroblastoma cells. The mice were kept in the rooms of the Tierforschungszentrum der Universität Ulm. They got water and dehydrated food ad libitum. Experiments were done with the approval (approval number 694) of the Regierungspräsidium Tübingen.

2.1.5.2 Drug preparation for in vivo experiments

For intraperitoneal injections cytosine was complexed with γ-cyclodextrin in a 1% lactic acid solution. The final cytosine concentration was 173 mM, the final concentration of γ-cyclodextrine was 63 mM.

For oral administration cytosine was added to the drinking water at a concentration of 1g /130 ml (= 69 mM).

N-(phosphonacetyl)-L-aspartate (PALA, MW 299.1) was dissolved in 0.9% sodium chloride to a final concentration of 5 mg PALA per 0.2 ml volume (= 84 mM).

For intraperitoneal injections, 12.5 mg 5-FC (MW 129.1) were dissolved in 600 µl PBS (= 161 mM) .

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Transformation and extraction of plasmid DNA

E. coli SCS110 were thawed on ice, 100 µl aliquots were put into prechilled Falcon tubes and 1.7 µl mercaptoethanol was added. The tubes were incubated on ice for 10 min and swirled gently every 2 min. Then SCS110 were incubated with 50 ng of plasmid DNA (pLXIN or pL(FCU1)IN), swirled gently and incubated on ice for 30 min. This mixture was then heat-pulsed in a water bath at 42℃ for 45 seconds and put immediately on ice for 2 min. The transformed bacteria were seeded on LB agar containing 100 µg ampicillin per ml. The agar plates were incubated at 37℃ over night. The next day, single colonies were picked by sterilized tips from the agar plates and inoculated into 3 ml LB medium containing ampicillin at a concentration of 100 µg per ml. The LB medium was incubated for 8 hours at 37℃ with vigorous shaking. 100 µl of bacteria culture were inoculated into 100 ml LB medium containing 100 µg ampicillin per ml and again incubated at 37℃ for 12 to 16 hours with vigorous shaking. Bacteria were harvested by centrifugation at 6000 x g for 15 min at 4℃. The pellet was resuspended completely in 10 ml resuspension buffer P1 containing RNAase A and then mixed with 10 ml of lysing buffer P2 thoroughly by inverting the tube 5 times and incubated for 5 min at RT. The lysate was then incubated with 10 ml prechilled neutralizing buffer P3 for 20 min on ice. The neutralized lysate was then centrifuged at 20000 x g for 30 min at 4℃. Supernatant was collected, filtered by Quiagen-tip and the DNA allowed to bind onto the resin. The Quiagen-tip was washed twice with 30 ml washing buffer QC and DNA was eluted with 15 ml elution buffer QF. DNA was precipated by adding 10.5 ml isopropanol and centrifugated at 15000 x g for 30 min at 4 ℃. The pellet was washed with 5 ml 70% ethanol and centrifuged at 15000 x g for 10 min at 4℃. The pellet was air-dried under sterile conditions for 5 to 10 min and the plasmid DNA redissolved in a suitable volume of TE buffer. DNA concentration was determined by UV spectrophotometry. DNA solution was stored at -20°.

2.2.1.2. Determination of DNA concentration by UV spectrophotometry

In order to determine DNA concentrations, the extinction of an aqueous DNA solution was measured at 260 nm wavelength, the maximum of absorption of double stranded DNA (ds DNA). One OD unit (optical density unit) corresponds to 50 µg/ml ds DNA. Since also other substances, like amino acids with aromatic side chains, have their maximum of absorption at this wavelength, this method is not completely specific. In order to get information about the purity of the DNA, the samples were also measured at 280 nm wavelength. The quotient of the OD at 260 nm (OD_{260}) / 280 nm (OD_{280}) wavelength was calculated. For pure DNA this quotient should be between 1.8 and 2.0.

2.2.1.3 Plasmid construction

In order to obtain a plasmid containing the desired therapeutic gene, the cytosine deaminase / uracil phosphoribosyltransferase fusion gene (FCU1) was excised from plasmid pTG 13046 and cloned into the multiple cloning site of the mammalian expression vector pLXIN.

2.2.1.3.1 Excising the cytosine deaminase / uracil phosphoribosyltransferase fusion gene from plasmid pTG 13046

The cytosine deaminase / uracil phosphoribosyltransferase fusion gene (FCU1) was excised from plasmid pTG 13046 as a 1189 bp fragment using XhoI and SmaI.

3 µg of plasmid pTG 13046 was incubated with 1 µl SmaI (10 U/µl) in a 20 µl buffered reaction system for 2 hours at 37℃. Restriction endonucleases were then inactivated at 65℃ for 20 min and the mixture was purified using the QIAquick PCR purification kit according the protocol supplied by the manufacturer in order to remove enzymes that are still active.

Blunt ends of the linearized plasmid DNA were then created by incubating the purified plasmid DNA with 2,5 µl Klenow Enzyme (2 U/µl) and 0,5 µl dNTP Mix (2 mM) in a 20 µl buffered reaction system at 37°C for 30 minutes.

The plasmid DNA was again purified using QIAquick PCR purification kit, incubated with 1 µl XhoI (10 U/µl) in a 20 µl buffered reaction system for 2 hours at 37℃ and heat inactivated at 65℃ for 20 min. The digested plasmid DNA was loaded with loading buffer and run on a 1% agarose gel at 12 V over night. DNA fragments were analyzed by UV light, and the desired 1189 bp fragment excised from the gel and purified using the QIAquick gel extraction kit according to the manufacturer´s instruction.

2.2.1.3.2 Linearizing and preparing pLXIN for insertion

3 µg of pLXIN was incubated with 1µl BamHI (10 U/µl) in a 20 µl buffered reaction system for 2 hours at 37℃. Restriction endonucleases were inactivated at 65℃ for 20 min, the mixture was purified using QIAquick PCR purification kit, then incubated with Klenow Enzyme as described above, again purified using the QIAquick PCR purification kit and incubated with 1 µl XhoI (10 U/µl) in a 20 µl buffered reaction system for 2 hours at 37℃ and heat-inactivated at 65℃ for 20 min as described above.

In order to prevent self-ligation in the following step (2.2.2.3), the purified linearized plasmid DNA was incubated with shrimp alkaline phosphatase according to the manufacturer´s instruction. Shrimp alkaline phosphatase was inactivated irreversibly by heat treatment for 15 min at 65℃ before purifying the linearized plasmid DNA on a 1% agarose gel as described above.

2.2.1.3.3 Insertion of the excised FCU1 fragment in the linearized pLXIN vector.

The purified insert (FCU1) was inserted in the purified linearized vector pLXIN using Rapid DNA Ligation Kit according to the manufactorer´s instruction in the following reaction system: 10 µl DNA (vector + insert, ratio 1:3) were diluted in 10 µl 2 x T4 DNA ligation buffer, mixed thoroughly and incubated with 1 µl T4 DNA ligase for 10 min at RT. The recombinant DNA was cut by the same endonucleases used for subcloning and electrophoresed on a 1% agarose gel to detect the target fragment. Finally, the recombinant DNA (called pL(FCU1)IN) was propagated and a maxipreparation was performed as described above.

2.2.1.3.4 Virus production and generation of stable transduced bulk cultures

3×10^5 cells of the pantropic packaging cell line GP-293 per well were plated on a 6-well-plate and incubated over night. The next day 12 µg of the plasmid pL(FCU1)IN, respectively pLXIN, and 3 µg of pVSV-G were mixed with 30 µl of GenePORTER, a liposomal transfection agent in 1ml serum-free medium and added to the cells.

Liposome mediated transfection works by binding of the negatively charged phosphate groups of DNA to the positively charged surface of the liposome. The mechanism by which these complexes cross the cell membrane is not yet fully understood. Liposome mediated transfection was chosen because it guarantees high levels of transfection on adherent cells.

After 12 hours the medium was removed and replaced with medium containing 10% calf serum and 3 µM Trichostatin A containing medium. Trichostatin A, a microbial metabolite, is an inhibitor of histone deacetylases which are known to repress transcription. After 12 hours, retroviral supernatants were removed, filtered trough a 0.45 µm cellulose acetate filter, diluted 1:5 with serum-containing medium and mixed with 8 µg / ml polybrene to transduce target cells. Polybrene, a small positively charged molecule, facilitates transduction by binding to the negatively charged cell surfaces. This neutralizes surface change and allows the viral glykoproteins to bind more efficiently to their receptors due to reduced repulsion between cell surface and the viral glykoproteins.

Stable bulk cultures of NXS2pLXIN and NXS2pL(FCU1)IN were generated using neomycin at a concentration of 0.7 mg per ml for 14 days.

2.2.2 Cell biology methods

2.2.2.1 Cell culture

NXS2, NXS2pLXIN and NXS2pL(FCU1)IN were plated on collagen coated tissue culture flasks or well-plates. Cells were maintained in completed DMEM medium and grown at 37℃ and 5% CO_2 in a humidified atmosphere. Culture medium was changed when it appeared yellow.

For passing, cells were washed once with PBS and treated with Trypsin/EDTA. The reaction was stopped by adding serum-containing medium. The cell suspension was then centrifuged at 1200 g for 5 min, the cell pellet was resuspended in fresh culture medium and split at an appropriate ratio for further culture.

For freezing, cells were harvested and resuspended in completed medium. The cell resuspension was mixed 1:1 with freezing medium and a 1.5 ml aliquot was transferred to 1.8 ml cryotubes, stored at 80℃ for 24 hours, then put in -179℃ liquid nitrogen for long term storage.

For thawing, the cryotube was removed from liquid nitrogen, promptly put into 37℃ water bath and thawed for 2 min. Cell s were then diluted in 10 ml completed medium, centrifuged, resuspended in completed medium and plated into culture flasks. Medium volume was 2 ml for 6-well-plates and 0.2 ml for 96-well-plates.

2.2.2.1.1 Drug sensitivity

1.5 x 10^6 cells were plated on collagen-coated 96-well-plates and incubated overnight. The following day, increasing concentrations of 5-FC, PALA and cytosine were added for 5 (5-FC) or 7 (PALA, cytosine) consecutive days. Viability was measured by MTT assay [4] with the survival of PBS treated controls set as 100%.

2.2.2.1.1.1 MTT assay

The MTT assay, first described by Mosemann in 1983, is a quantitative colorimetric method to determine cell survival. It is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of MTT and form purple formazan crystals within healthy cells. Solubilisation of the cells by the addition of a detergent results in liberation of the crystals which are solubilized. The number of viable cells is directly proportional to the level of the formazan product created. The color can then be quantified using a multi-well scanning plate reader.

For MTT assay cells cultured in 96 well plates were carefully washed with 37℃ warm DMEM without phenol red. 100 µl MTT working solution was added into each well and plates were again incubated at 37℃ for one hour. At the end of the incubation period the medium was removed carefully and 100 µl isopropanol was added to solubilize the converted dye. The isopropanol in each well was pipetted up and down several times to ensure the converted dye dissolves completely.

The absorbance of the converted dye was measured at a wavelength of 570 nm using a multiwell plate reader (ELx 800) (Bio-Tec Instruments, Germany).

2.2.2.2 Modulation of PALA-induced cytotoxicity by cytosine

1.5 x 10^6 cells were plated on collagen-coated 96-well-plates, incubated overnight, and treated with 12.5 or 25 µM PALA in the presence of increasing concentrations of cytosine for 7 consecutive days. Viability was measured by MTT assay as described above.

2.2.2.3 Determination of doubling time

NXS2 and its derivatives were seeded at a density of 1 x 10^5 in 5 ml completed DMEM on 25 cm² collagen coated tissue culture flasks. When the cells reached a density of about 80% confluence, cells were trypsinized and the cell number was determined using a Neubauer counting chamber.

A Neubauer counting chamber is used to count cells in suspension by observing through a microscope a calibrated grid of which the exact dimensions

(including depth and volume) are known. The number of cells per ml can be then determined by a simple calculation. A coverslip is placed over the counting surface prior to putting on the cell suspension. When the cell suspension is introduced with a pipette, the area under the coverslip fills by capillary action. In order to get a statistically significant count, all four large corner squares on the Neubauer counting chamber were counted. This procedure was repeated twice.

Generation time for each cell line was determined according to the following formula:

Generation time = t_g= (log2 x t) / (logN – logN_0).

t = hours between seeding and counting

N = number of cells after cultivation for time t

N_0 = number of cells seeded = 1 x 10^5

2.2.2.4 DiI labeling of cells

DiI is a relatively inert lipophilic tracer that stains cell membranes by diffusing along the lipid bilayer. Advantage of DiI are its low toxicity and the fact that it is retained in cells for long periods of time (even through cell division).

DiI was dissolved in absolute ethanol to a final concentration of 10 mg per ml and stored in amber bottles at room temperature. For staining, cells were plated in DMEM containing 0.1 mg per ml of the lipid soluble fluorescent dye DiI, incubated for 60 minutes at 37°C, washed once with completed DMEM and rinsed daily for two days before experimentation.

2.2.2.5 In vitro enrichment

DiI labeled NXS2pL(FCU1)IN were mixed with NXS2 cells in increasing ratios. 1.0 x 10^4 cells were incubated overnight in collagen-coated 6-well-plates and treated with 12.5 µM PALA and 5 mM cytosine or PBS alone. After 7 days DiI positive cells in the mixture were counted using FACS analysis (2.2.2.6.1) and analyzing 2 x 10^4 cells without gating. Unstained NXS2 cells were used as a negative control. In order to see whether treatment with PALA and cytosine will lead to an increase in the relative share of NXS2pL(FCU1)IN cells in the mixture, PALA and cytosine treated samples were compared to PBS treated samples.

2.2.2.5.1 FACS analysis

Fluorescence activated cell sorting (FACS) allows the isolation of defined cell subsets from heterogeneous mixtures. FACS uses laser light to excite fluorescent labeled molecules and subsequently detects the emitted fluorescence on cells passing in single file in a fluid stream. The fluorescent labeled molecules can be either attached to or located inside cells. The advantage of FACS is that it allows the measurement of the fluorescence on thousands of individual cells.

We used the lipophilic tracer DiI (2.2.2.5) to stain NXS2pL(FCU1)IN in order to distinguish them from wild type NXS2 cells by FACS. For FACS analysis cells were trypsinized, centrifuged at 1200 g for 5 min, the pellet was resuspended in FACS buffer and the cellular fluorescence was detected on a FACS-Calibur Instrument at channel 2 (488 nm wavelength) using CellQuest software and analyzing 20 000 events without gating.

2.2.2.6 In vitro bystander effect

NXS2pL(FCU1)IN were mixed with NXS2 cells in increasing ratios. 1.5 x 10^3 cells were seeded in collagen coated 96-well-plates, incubated overnight and exposed to 1 mM 5-FC for 5 days. Viability was measured by MTT assay as described above.

2.2.2.7 In vitro enrichment – eradication

NXS2pL(FCU1)IN and NXS2 cells were cocultivated with increasing ratios and enriched with 12.5 µM PALA and 5 mM cytosine for 7 days or left untreated. On day 7 cells were trypsinized and (to prevent overgrowth) replated at a density of 1.5 x 10^3 in collagen-coated 96-well-plates and exposed to 1 mM 5-FC for 5 days. Viability was measured by MTT assay as described above.

2.2.2.8 Modulation of 5-FC sensitivity by pretreatment with PALA

NXS2pL(FCU1)IN were incubated with 12.5 µM PALA and 5 mM cytosine or PBS for 6 consecutive days. On day 6 cells were trypsinized, counted and replated at a density of 1500 cells per well on 96-well-plates. Cells were incubated over night and then exposed to increasing concentrations of 5-FC for 5 days. Viability was measured by MTT assay as described above.

2.2.3 Animal methods

2.2.3.1 Cytosine pharmacokinetics

6-8 week old female A/J mice were injected i.p. with 173 mM cytosine dissolved in 500 µl aqua bidest. or complexed with γ-cyclodextrin in 500 µl lactic acid solution. In order to prepare cytosine / γ-cyclodextrin complexes cytosine (173mM) and γ-cyclodextrin (63mM) were dissolved in a 1% lactic acid solution to yield a cytosine / γ-cyclodextrin complex solution. 0.5, 1, 2, 4, 6 and 7 h after injection of cytosine or cytosine / γ-cyclodextrin complexes 0.5 to 1.0 ml blood was aspirated by cardiac puncture, plasma was separated promptly by centrifugation and plasma samples were immediately stored on dry ice and kept at –80°C until analysis. For each time point, the plasma of two mice was pooled to have a volume sufficient for analysis. Cytosine plasma levels were determined by HPLC. HPLC analyzes of cytosine plasma levels were done by Berthold Büchele and Thomas Simmet at the Institut für Klinische Pharmakologie, Universität Ulm.

2.2.3.2 In vivo enrichment

6 % DiI labeled NXS2pL(FCU1)IN were mixed with 94 % unlabeled NXS2 cells.
2 x 10^6 cells of this mixture were injected subcutaneously into 6-8 week old female A/J mice. On day 3, when tumors were palpable, mice with no or with advanced tumors were sacrificed. All other mice were randomly distributed into two groups. The enrichment group (n=5) received 3 doses of 5 mg PALA in 200 µl 0.9 % sodium chloride i.p. (days 3, 6, 10) and daily i.p. injections of 86.5 µmol cytosine / γ-cyclodextrin complexes in 500 µl of 1.0 % lactic acid buffer. To further increase

cytosine delivery, cytosine was also added to the drinking water of this group at a concentration of 1g / 130 ml. The control group (n=5) received daily i.p. injections of 500 µl of 1.0 % lactic acid buffer only. When tumors reached a diameter of 8 mm, animals were sacrificed by cervical dislocation, tumors were explanted, cryopreserved and cryosectioned at –25℃ (CM 1850, Leica Microsystems, Nussloch, Germany). Serial sections 4 µm in thickness were made and mounted on microscope slides. The sections were counterstained by hematoxylin and assessed using a fluorescent microscope (AX 70, Olympus, Stuttgart, Germany). Hematoxlyin staining was used instead of the routine hematoxylin and eosine (H&E) stain in order not to vanish DiI. Pictures were taken with a CC-12 camera and digitally processed (Soft Imaging System, Muenster, Germany).

2.2.3.3 In vivo enrichment and eradication

6-8 week old female A/J mice (Harlan) were injected subcutaneously with 2×10^6 cells containing 6 % NXS2pL(FCU1)IN and 94 % NXS2. 3 days later, when tumors were palpable, treatment was started. Mice with no or with advanced tumors were sacrificed. All other mice were randomly distributed into two groups. The control mice received daily injections of 500 µl 1% lactic acid buffer from day 3 to 12. Cytosine was given orally at a concentration of 1 g per 130 ml in the drinking water and additionally as daily i.p. injections of 500 µl cytosine / γ-cyclodextrin solution. PALA was administered three times as i.p. injection of 5 mg in 200 µl 0.9% sodium chloride solution. 5-FC was given daily as i.p. injection of 12.5 mg in 600 µl PBS. Tumor size was measured every second day and tumor volume calculated as volume = longest diameter x [shortest diameter] ^{2} x 0.4. Mice were killed by cervical dislocation when they appeared moribund. Survival was analyzed using Kaplan-Meier plotting and significance was tested by the log rank test.
Values of $p < 0.05$ were considered significant.

3. Results

3.1 Sensitivity of NXS2 and its derivatives to 5-FC, PALA and cytosine

Since the sensitivity of NXS2 cells to 5-FC, PALA and cytosine is unknown we exposed wild type NXS2 and its derivatives to 5-FC, PALA and cytosine. The prodrug 5-FC killed suicide gene transduced NXS2pL(FCU1)IN depending on dose. Mock-transduced NXS2pLXIN and wild type NXS2 (Fig.4 A) were spared, since these cells were not able to metabolize the non-toxic prodrug due to the lack of cytosine deaminase. Thus, 5-FC induced cell death was selective for NXS2 pL(FCU1)IN within a broad therapeutic range.

Incubation of NXS2pL(FCU1)IN, NXS2pLXIN and NXS2 with increasing concentrations of the competitive enzyme inhibitor PALA for 7 consecutive days demonstrated an equal degree of cytotoxicity in all three tested cell derivatives in a MTT assay (Fig.4 B). This indicates, that the sensitivity to this specific pyrimidine de novo synthesis inhibitor is not altered by the FCU1 gene product.

When NXS2 and its derivatives were exposed to increasing concentrations of the pyrimidine base cytosine for 7 consecutive days no toxicity was observed (Fig. 4 C). This shows, that cytosine can be used in the proposed enrichment strategy without exerting untoward effects of its own.

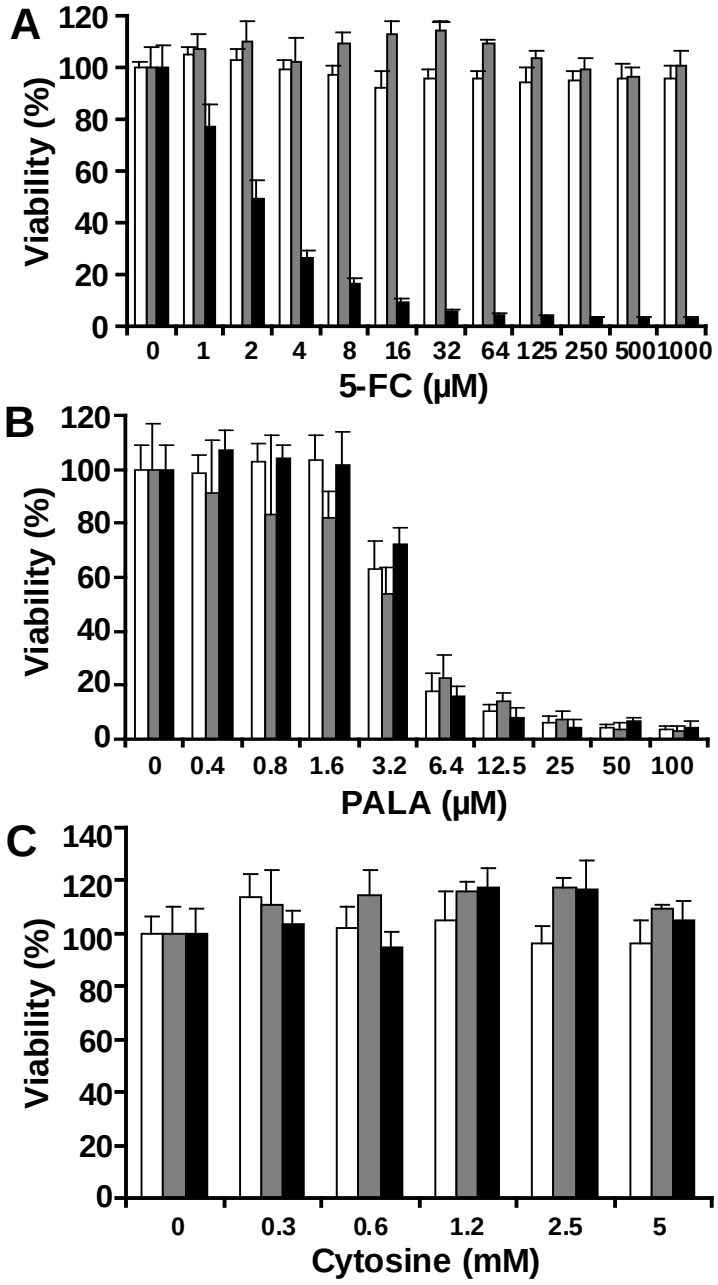

Fig. 4 Sensitivity of NXS2□ , NXS2pL(FCU1)IN▒ and NXS2pL(FCU1)IN■ to 5-FC, PALA and cytosine. Cells were exposed to increasing concentrations of 5-FC, PALA or cytosine for 5 days (5-FC) or 7 days (PALA and cytosine). Survival was measured by MTT assay. Results are means of quintuplicates and are expressed as percentage of PBS treated controls. Similar results were obtained in three independent experiments for each drug. (**A**) NXS2pL(FCU1)IN are efficiently and selectively killed by 5-FC in a dose-dependent manner. (**B**) NXS2 derivatives are equally sensitive to PALA. (**C**) Cytosine is not toxic to NXS2 derivatives.

3.2 Cytosine attenuates PALA-induced cytotoxicity selectively in FCU1 expressing cells

To determine whether FCU1 expressing cells can be selectively rescued from PALA-induced cytotoxicity by sole addition of cytosine, NXS2pL(FCU1)IN, NXS2pLXIN and NXS2 were exposed to 12.5 and 25 µM PALA in the presence of increasing concentrations of cytosine. These concentrations of PALA were chosen because they kill a large percentage (about 80%) of all three NXS2 derivatives in vitro when no cytosine is added (see Fig. 4 B). Indeed, increasing concentrations of cytosine resulted in increased survival of NXS2pL(FCU1)IN (Fig. 5). When the cytosine concentration reached 10 mM NXS2pL(FCU1)IN cells were virtually protected from the cytotoxic effects of PALA. In contrast, sensitivity of NXS2pLXIN and wild type NXS2 to PALA was not altered by cytosine even at high concentrations. These data show, that cytosine selectively and efficiently attenuates PALA-induced cytotoxicity in NXSpL(FCU1)IN cells.

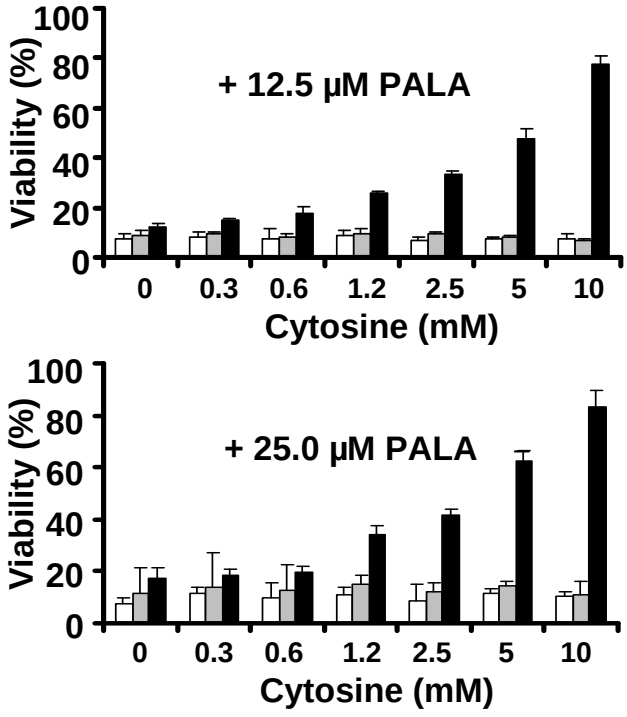

Fig. 5 Cytosine attenuates PALA induced cytotoxicity selectively in NXS2 cells that express FCU1. NXS2□ NXS2pLXIN■ and NXS2pL(FCU1)IN■ were treated for 7 days with PALA (12.5 or 25 µM) in the presence of cytosine in the concentrations indicated. Survival was measured by MTT assay. Results are means of quintuplicates and are expressed as percentage of PBS treated controls. Similar results were obtained in three independent experiments.

3.3 PALA and cytosine effectively enrich for FCU1 expressing cells

To investigate whether the protective effect of cytosine for FCU1 expressing cells incubated with PALA is effective enough to enrich these cells, a cocultivation experiment with wild type tumor cells was performed. NXS2pL(FCU1)IN labeled with DiI were mixed with unlabeled NXS2 and exposed to 12.5 µM PALA and 5 mM cytosine for 7 days. These concentrations of PALA and cytosine were chosen because they kill about 80% of NXS2, while more than 60% of NXS2pL(FCU1)IN cells survive (see Fig. 5). The percentage of DiI-labeled NXS2pL(FCU1)IN was determined by FACS analysis on day 7. The proportion of NXS2pL(FCU1)IN was markedly increased by treatment with PALA and cytosine, whereas the ratio remained unchanged in the PBS-treated controls (Fig. 6). This enrichment of NXS2pL(FCU1)IN was not due to differences in proliferation since no relevant difference in doubling time was observed (Tab. 1). Thus, one of the prerequisites of the proposed strategy, namely an efficient enrichment of the suicide gene-bearing cells, was met.

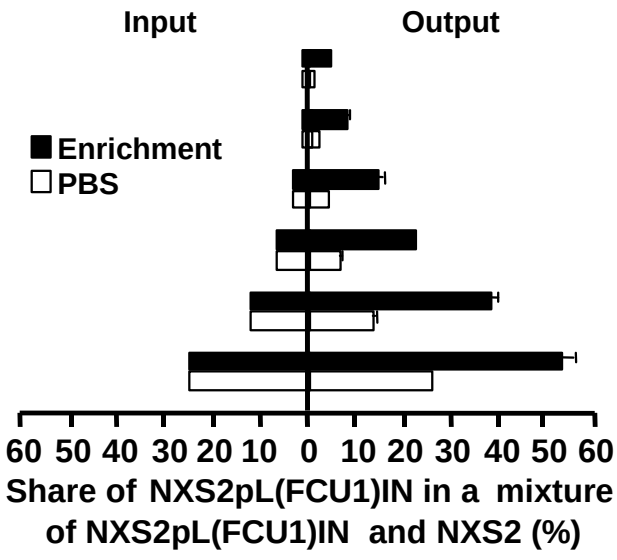

Fig. 6 PALA and cytosine select for FCU1 expressing cells. NXS2pL(FCU1)IN labeled with DiI were mixed with NXS2 cells in shares indicated and treated with PALA (12.5 µM) in the presence of cytosine (5 mM)■. After 7 days, the percentage of DiI positive cells, i.e. NXS2pL(FCU1)IN, was determined by FACS analysis. Results are means of triplicates and are expressed as percentage of PBS treated controls□. Similar results were obtained in two independent experiments.

Tab.1 Doubling time of NXS2 and ist derivatives. NXS2 and its derivatives exhibit no relevant differences in doubling time. Mean values of doubling time and standard deviations are shown in hours (h).

Cell line	NXS2	NXS2pLXIN	NXS2pL(FCU1)IN
Doubling time in h	21.00	21.40	21.35
Standard deviation	± 0.57	± 0.00	± 0.07

3.4 FCU1 / 5-FC exerts a very strong bystander effect in vitro

Another sine qua non of the proposed strategy is a strong bystander effect, i.e the killing of non-transduced cells in the surrounding of transduced cells due to diffusion of cytotoxic metabolites via membranes. In a cocultivation experiment with wild type NXS2 we therefore determined the percentage of NXS2pL(FCU1)IN necessary for a complete bystander effect in vitro. 12.5 % of NXS2pL(FCU1)IN sufficed to eradicate all tumor cells via the bystander effect (Fig. 7). These data suggest, that a small enrichment of transduced cells may suffice to control tumor cell growth.

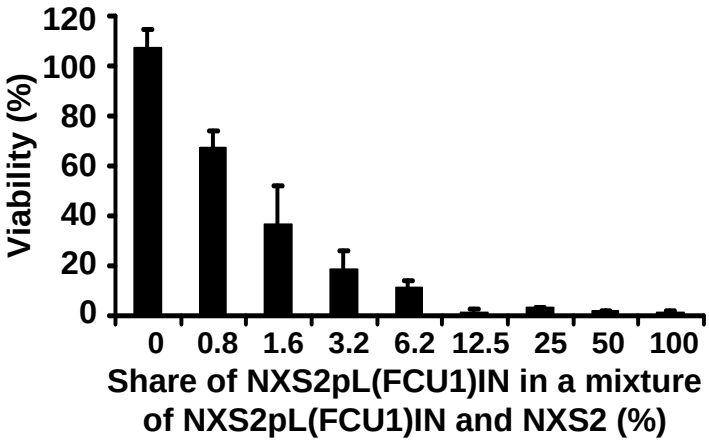

Fig. 7 5-FC / FCU1 exerts a very strong bystander effect in vitro. NXS2pL(FCU1)IN and NXS2 were cocultivated at the ratios indicated and exposed to 5-FC (1 mM) for 5 days. Survival was measured by MTT assay. Results are means of quintuplicates and are expressed as percentage of PBS treated controls. Similar results were obtained in three independent experiments.

3.5 Enrichment of NXS2pL(FCU1)IN results in a near-complete bystander effect in vitro

To test the notion, that enrichment by PALA and cytosine may suffice for a complete bystander effect, even if only a small fraction of cells carry FCU1 initially, we performed a two step enrichment-eradication experiment. NXS2pL(FCU1)IN and NXS2 were seeded at a ratio of 0.8 % and 1.6 %. This low percentage of suicide gene-bearing cells in the mixture was chosen in order to mimic the range of gene transfer efficiency in vivo. This proportion of transduced tumor cells kills about 50% of all tumor cells in an in vitro bystander setting (Fig. 7). Indeed, enrichment by PALA and cytosine allowed for near-complete eradication of all tumor cells upon 5-FC treatment (Fig.8), whereas the bystander effect was incomplete in the absence of enrichment. This near-complete bystander effect after enrichment was mainly due to an increased number of FCU1-bearing tumor cells and was not caused by pharmacological cooperative effects between the two drugs, since PALA alone only marginally modulated the sensitivity of NXS2pL(FCU1)IN cells to 5-FC (Fig.9).

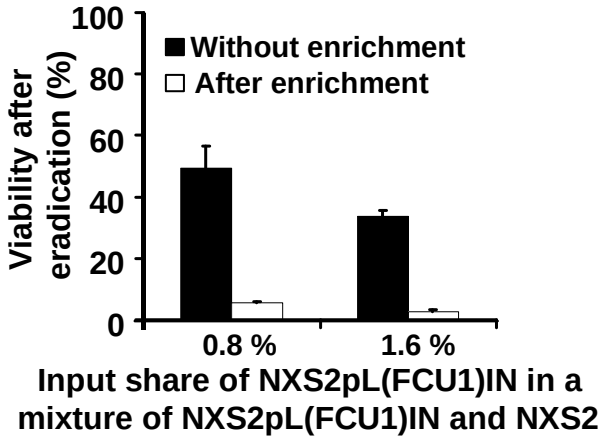

Fig. 8 Enrichment allows for subsequent eradication of tumor cells. Efficient enrichment of low shares of NXS2pL(FCU1)IN allows for a near-complete bystander effect. NXS2pL(FCU1)IN and NXS2 were cocultivated at the ratios indicated and were enriched with PALA and cytosine for 7 days or were not enriched. On day 7 cells were replated to prevent overgrowth and were exposed to 5-FC (1 mM) for 5 days. Viability was then measured by MTT assay. Results are means of quintuplicates and are expressed as percentage of PBS-treated controls. Similar results were obtained in three independent experiments.

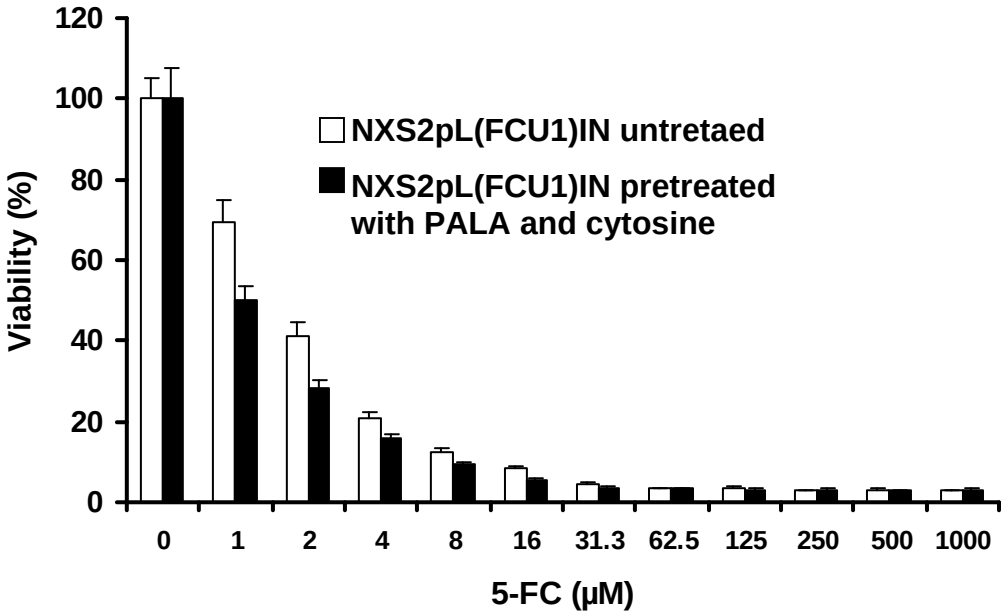

Fig. 9 PALA alone only marginally sensitizes NXS2 cells to FCU1 / 5-FC. NXS2pL(FCU1)IN were pretreated with 12.5µM PALA for 6 days or left untreated and were then exposed to increasing concentrations of 5-FC. Viability was measured by MTT assay. Results are means of quintuplicates and are expressed as percentage of PBS-treated controls.

3.6 High and prolonged cytosine plasma levels can be achieved in vivo

To show that the high cytosine concentrations required for effective rescue in vitro can also be reached in vivo we, in cooperation with the Institut für Klinische Pharmakologie, conducted pharmacokinetic studies in female A/J mice. Caged in γ-cyclodextrin, a carbohydrate molecule that improves the solubility of cytosine and slows down its excretion in vivo, cytosine reached peak plasma levels of 2 mM and exhibited a half-life of about 100 minutes. No acute toxicity was noted during the observation period nor were gross signs of toxicity observed during autopsy. Thus, cytosine in γ-cyclodextrin is suitable for in vivo enrichment.

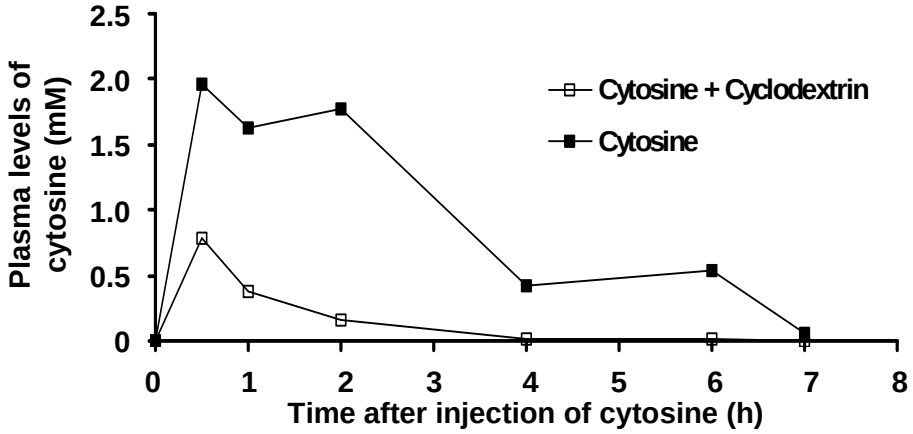

Fig. 10 Increased and prolonged plasma levels of cytosine by complexing with γ-cyclodextrin. 6-8 week old female A/J mice were injected i.p. with 70 mM cytosine dissolved in 500 µl water or complexed with γ-cyclodextrin in 500 µl lactic acid solution. Following injection mice were killed at the time points indicated and 0.5 to 1.0 ml blood was aspirated by cardiac puncture. Plasma cytosine levels were determined by HPLC.

3.7 NXS2pL(FCU1)IN can be enriched in vivo

To determine, whether enrichment is feasible in vivo, a small number of DiI-labeled NXS2pL(FCU1)IN mixed with a surplus of NXS2 was injected s.c. into mice. Palpable tumors were treated with PALA and cytosine for seven days. NXS2pL(FCU1)IN became markedly enriched, growing in clusters surrounded by NXS2, whereas the initial percentage of NXS2pL(FCU1)IN remained constant in control tumors (Fig. 11).

This shows, that enrichment is feasible not only in monolayer cell culture but also in three-dimensional growing tumors. Weight loss during enrichment did not exceed 15% of body weight, no mice died during enrichment and no overt signs of toxicity were noted by autopsy, ruling out life-threatening adverse effects of enrichment.

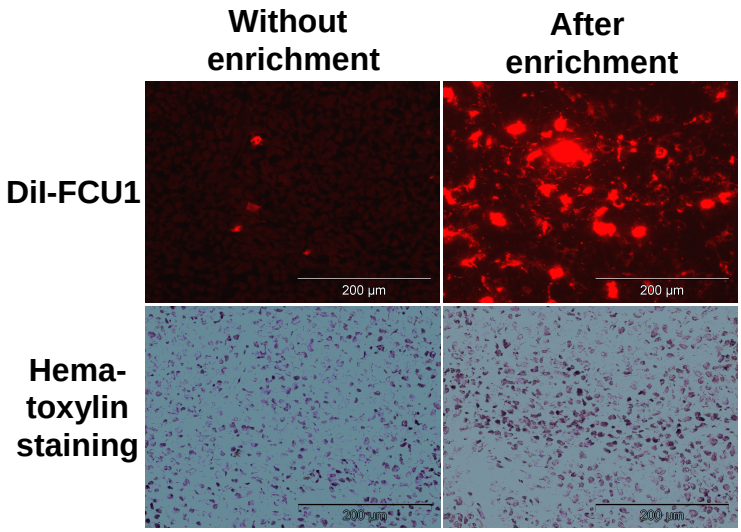

Fig.11 PALA and cytosine enrich for NXS2pL(FCU1)IN in vivo. A mixture of 6% DiI labeled NXS2pL(FCU1)IN labeled and 94% non-transduced NXS2 cells was injected subcutaneously into mice. Mice were subjected to enrichment therapy with PALA and cytosine (right panels) or were sham treated with solvent alone (left panels). Tumors were excised once a volume of 200 μm^3 was reached and analyzed by fluorescence (upper panels) and light microscopy (lower panels).

3.8 The enrichment-eradication strategy decreases tumor growth and increases survival of mice

Finally we wanted to know, whether the enrichment-eradication strategy functions in vivo. Based on preliminary experiments, 10 days were chosen for both the enrichment and the eradication block of this strategy. "Early" eradication (5-FC for the first ten days followed by ten days without drugs) was used for comparison with "inverse" therapy (5-FC for the first ten days followed by ten days of PALA). "Late" eradication (10 days without drugs followed by 5-FC for 10 days) served as comparison for enrichment-eradication (PALA for 10 days and 5-FC during the last ten days). We observed a clear trend towards slowest tumor growth in the mice receiving enrichment-eradication therapy (Fig. 5A). Tumor growth was slower compared to "late" (and "early") eradication and also compared to "inverse" therapy. The latter rules out a mere additive effect of the enrichment and eradication blocks. Interestingly, PALA alone was ineffective and did not add to the effect of 5-FC during "inverse" therapy since "inverse" therapy was not superior to "early" eradication.

Survival analysis of the mice revealed the same pattern as observed with tumor growth. Enrichment-eradication led to significant superior survival compared to "inverse" therapy and both "early" and "late" eradication, which in turn showed significantly prolonged survival compared to the untreated control group (Fig. 5B). No effect was seen when PALA was given alone. Survival of cytosine-treated mice did not differ from control mice, ruling out life-threatening adverse effects of cytosine.

Taken together, these data show that enrichment of a low percentage of FCU1-bearing cells followed by eradication decreases tumor growth and increases survival without severe toxicity.

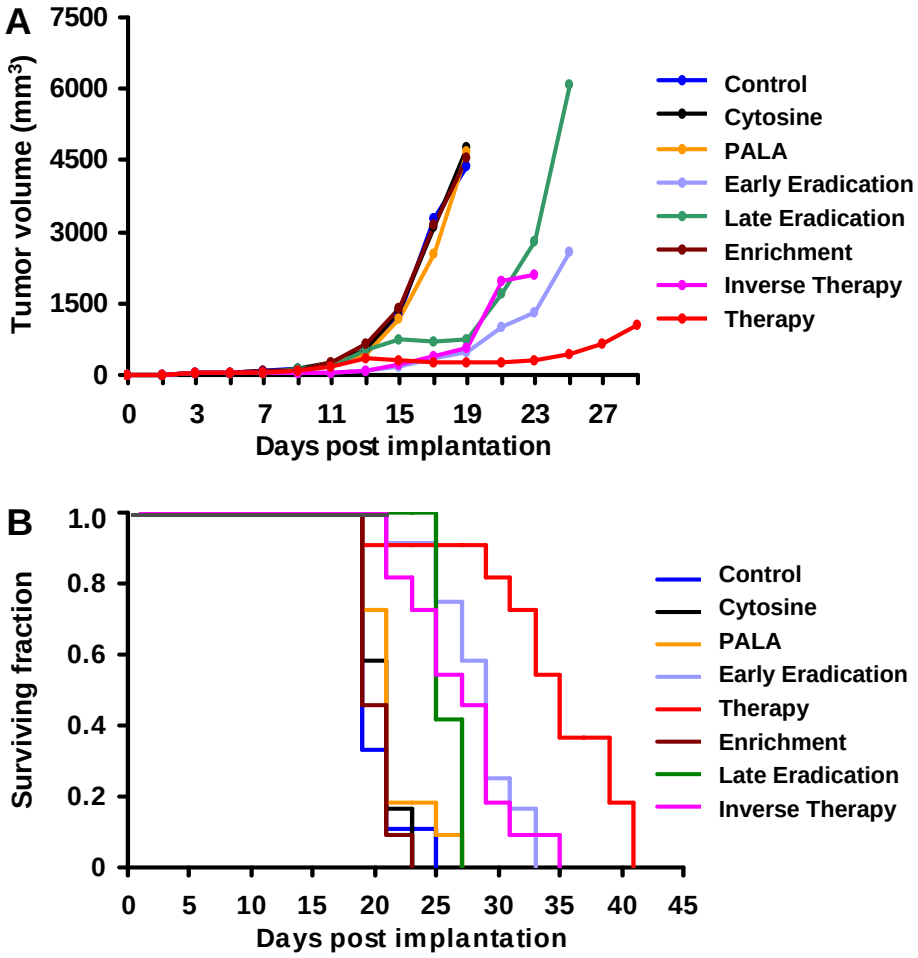

Fig. 12 The two-step enrichment – eradication strategy decreases tumor growth and prolongs survival of tumor-bearing mice. (A) Enrichment-eradication decreases tumor growth more than eradication alone or eradication preceding enrichment. NXS2pL(FCU1)IN were mixed with non transduced NXS2 at a ratio of 1 to 16 (i.e. 6.25% NXS2pL(FCU1)IN) and injected subcutaneously in mice. Three days later mice were randomly distributed to the following groups. *Control* group (n=9): 1% lactic acid buffer i.p. from day 3 after injection to day 12. *Cytosine* group (n=12): cytosine orally and i.p. from day 3 to day 12. *PALA* group (n=11): PALA i.p, on day 3, 6 and 10. *Early Eradication* group (n=12): 5-FC from day 3 to day 12. *Late Eradication group* (n=12): 5-FC from day 13 to 22. *Enrichment* group (n=11): Cytosine from day 3 to day 12 and PALA on day 3, 6,10. *Inverse Therapy* group (n=11): 5-FC from day 3 to day 12 followed by cytosine from day 13 to day 22 and PALA on day 13, 16 and 20. *Therapy* group (n=11): Cytosine from day 3 to 12, PALA on day 3, 6 an 10 and 5-FC from day 13 to 22. Standard variations overlap and are shown in Tab. 2 for clarity. (B) The enrichment - eradication strategy prolongs survival significantly more than eradication therapy alone. The survival of the mice described in (B) is depicted as a Kaplan-Meier plot. Significance was determined by the log rank test. p-values were 0.0021 for *Therapy* versus *Inverse Therapy*, < 0.0016 for *Therapy* vs. *Early Eradication*, < 0.0001 for *Therapy* vs. *Late Eradication*, < 0.0001 for *Therapy* vs. *Control* , < 0.0001 for *Therapy* vs. *Enrichment*, 0.0003 for *Inverse Therapy* vs. *Control*, < 0.0001 for *Early Eradication* vs. *Control and* < 0.0001 for *Late Eradication* vs. *Control*.

Tab.2 Supplementary information for Fig. 12 A. Means ± standard deviations and number of animals per group for each time point. The mean tumor volume ± standard deviation in mm^3 and the number (n) of mice per group are given for each time point. Day 0 marks injection of tumor cells.

	Day 0	Day 5	Day 11	Day 15	Day19	Day 21	Day 23	Day 25
Control	0 ± 0 n = 9	49 ± 0 n = 9	216 ± 124 n = 9	1289 ± 853 n = 9	4366 ± 2195 n = 9	5516 ± 3005 n = 3	2884 ± 0 n = 1	5833 ± 0 n = 1
Cytosine	0 ± 0 n = 12	49 ± 0 n = 12	283 ± 127 n = 12	1348 ± 929 n = 12	4371 ± 2025 n = 12	5550 ± 1801 n = 7	6260 ± 1344 n = 9	
PALA	0 ± 0 n = 11	49 ± 0 n = 11	271 ± 147 n = 11	1886 ± 666 n = 11	4670 ± 2783 n = 11	6384 ± 3348 n = 8	5253 ± 2653 n = 5	5047 ± 371 n = 2
Enrichment	0 ± 0 n = 11	49 ± 0 n = 11	283 ± 136 n = 11	1385 ± 427 n = 11	4572 ± 1786 n = 11	6115 ± 1503 n = 5	5833 ± 0 n = 1	
Early Eradication	0 ± 0 n = 12	53 ± 14 n = 12	65 ± 24 n = 12	176 ± 125 n = 12	480 ± 411 n = 12	1017 ± 946 n = 12	1294 ± 1225 n = 11	2580 ± 2565 n = 11
Early Eradication	0 ± 0 n = 12	49 ± 0 n = 12	187 ± 82 n = 12	745 ± 262 n = 12	765 ± 336 n = 12	1718 ± 863 n = 12	2788 ± 1636 n = 12	6078 ± 3239 n = 12
Inverse Therapy	0 ± 0 n = 11	49 ± 0 n = 11	62 ± 23 n = 11	207 ± 142 n = 11	557 ± 312 n = 11	1990 ± 1965 n = 11	2108 ± 1771 n = 9	3441 ± 3052 n = 8
Therapy	0 ± 0 n = 11	49 ± 0 n = 11	165 ± 96 n = 11	328 ± 222 n = 11	277 ± 171 n = 11	259 ± 150 n = 10	293 ± 202 n = 10	421 ± 292 n = 10

4. Discussion

Poor in vivo gene transfer remains a major obstacle to the implementation of cancer gene therapy [5, 12, 19, 47, 48, 52, 61]. We provide first time evidence, that in vivo enrichment of suicide gene bearing tumor cells post gene transfer is an effective alternative to increasing gene transfer for improving tumor control.

We chose the murine neuroblastoma cell line NXS2 as a model to proof the principle of the suggested enrichment-eradication strategy. NXS2 cells are syngeneic to A/J mice [32] which allowed us to do in vivo experiments in immuno-competent mice. This seemed to be particularly important for us since the immune system modulates any kind of cancer therapy.

The proposed therapy consisted of 2 steps: increasing the share of suicide gene bearing tumor cells (enrichment) and subsequent eradication with the prodrug 5-FC.

Enriching tumor cells in order to treat a tumor is counterintuitive until the rationale of this approach is understood: first, the enrichment does not enhance tumor growth of suicide gene bearing tumor cells, but preferentially blocks the proliferation of non-transduced tumor cells. Second, the very strong bystander effect achieved after enrichment outweighs the initial decrease in tumor cell kill, thus leading to a net increase of tumor cell kill. Modifying the CD / 5-FC plus PALA system by incorporating UPRT enabled us to employ, for the first time, a suicide gene for positive selection in vivo. Yeast CD appeared especially well suited for an enrichment-eradication approach since its gap junction-independent negative selection function is well established and superior to the bacterial CD. Furthermore, CD acts upon the pyrmidine metabolism, which is amenable to manipulations leading to positive selection. Another advantage of the CD / 5-FC system is the long half-life of the activated drug [19] supporting the effective killing of tumor cells.

Suicide gene bearing NXS cells could be rescued from PALA induced cell death by addition of cytosine, allowing us to increase the relative share of NXS2pL(FCU1)IN in a mixture with NXS2. This enrichment was not due to an unspecific resistance or differences in proliferation. Yet, very high cytosine levels were necessary to see a maximal protective effect in vitro. Even though cytosine exhibited no toxicity in vitro, it was not clear whether the maximal achievable

cytosine plasma levels would be high enough to see this protective effect also in vivo. Addition of γ-cyclodextrin improved the solubility of cytosine and allowed to reach higher plasma levels and a prolonged half-life of cytosine in vivo. Yet, it was not possible to achieve high steady-state plasma levels of the drugs in vivo, since all drugs were delivered by pulse i.p. injections. However, continuous high plasma levels might be especially important not only for the protective effect of cytosine in the enrichment step, but also for the 5-FC killing in the eradication step. This might explain in part why the clear-cut effect that was seen in the in vitro experiments was not so evident in the in vivo studies. Continuous infusion of these drugs, which might be crucial for the success of the proposed strategy, poses technical problems in mice, but could be done in patients.

Enrichment led to clusters of suicide gene bearing NXS2 cells that were inhomogeneously distributed inside the tumor, whereas in the in vitro experiments NXS2 and NXS2pL(FCU1)IN were more evenly distributed. This different pattern of distribution might explain the differences between the BE in vitro and in vivo, even though the relative shares of the cells were the same.

While the CD-mediated enrichment-eradication strategy was successful in decreasing tumor growth and prolonging survival, it did not lead to cures. Several factors may account for this. First, while PALA was efficient in enriching suicide gene-bearing cells in tumors that were still small, it was ineffective in controlling tumor growth once tumors grew larger. It has been shown in patients that PALA decreases tumor blood flow thus decreasing delivery of this and other drugs to the tumor [23]. Thus, while small tumors may still be accessible to PALA via diffusion, larger tumors, which are dependent on tumor vessels, may not be. Second, as discussed before, high steady-state plasma levels of cytosine during enrichment may be more efficacious than the intermittent peaks of cytosine following bolus administration. The same holds true for 5-FC in the eradication step. Third, cytosine plasma levels after enrichment may still have been sufficiently high during the initial eradication phase to compete with 5-FC for CD, resulting in lower 5-FU production. Forth, a rebound was detected in tumors that initially showed a delayed tumor growth. This might be due as well to the end of therapy on day 22 as to the killing of FCU1 expressing cells in the tumor. Fifth, there is evidence that there is a reduction of 5-FU toxicity in hypoxic tumors [19, 18]. Finally, the rapid growth of the murine tumors poses problems of time constraints for both the

enrichment and eradication phase. This is in contrast to patient tumors which grow slower.

Several approaches can be envisioned to improve the CD system for enrichment-eradication. PALA may be substituted by other inhibitors of pyrimidine de novo synthesis that are more efficient in controlling growth of bystander cells in vivo. Also, genes more efficient than UPRT may be used to bypass rate-limiting steps of the pyrimidine salvage pathway, ideally obviating the need for exogenous cytosine. While the high plasma level of cytosine needed for rescue did not lead to adverse effects in the mice, its effects in humans are unknown. Finally, the bystander effect may be augmented after enrichment by other treatment modalities known to enhance the effects of CD/5-FC such as irradiation[22, 60].

Clinical application of the enrichment-eradication strategy would require in vivo transfer of the suicide gene. While we have modeled the in vivo experiments to simulate the low gene transfer efficacy of current delivery methods, further studies have to assess the performance of this strategy following in vivo gene delivery, addressing in particular intratumoral or intermetastases heterogeneity of enrichment.

Enrichment-eradication is potentially most promising for non-viral gene transfer systems into tumors since these heavily depend on an efficient bystander effect. However, enrichment-eradication following gene transfer by viruses (including non-oncolytic tumorspecific replicating viruses) may also be feasible.

An attractive feature of enriching cells in vivo using a suicide gene is the fail-safe mechanism of this approach since increased resistance is invariably linked with increased susceptibility to suicide gene-induced cell death. In addition, because both resistance and susceptibility are mediated by the same gene, the risk of conferring only resistance to tumor cells by in vivo recombination is nil. Because of its safety, enrichment-eradication may be employed for the selection of adult stem or progenitor cells, both hematopoietic and non-hematopoietic, with the possibility of complete eradication if untoward effects like transformation should occur or to titrate a beneficial state of mixed chimerism of genetically modified and endogenous stem or progenitor cells.

In conclusion then, enrichment-eradication increases the number of suicide gene-bearing tumor cells and improves tumor control and could thus complement efforts to increase gene transfer efficacy in cancer gene therapy.

Summary

The low efficacy of gene transfer severely limits the clinical implementation of cancer gene therapies requiring in vivo transfer. We now show, that in vivo enrichment of tumor cells post suicide gene transfer is an effective alternative to increasing gene transfer for enhancing tumor control. Enrichment allows for a very strong bystander effect after administration of the prodrug, leading to eradication of most tumor cells. For this enrichment-eradication strategy we used a suicide fusion gene comprised of cytosine deaminase from yeast and uracil phosphoribosyl-transferase (FCU1). This suicide gene is bifunctional since it allows for both positive and negative selection. Positive selection (enrichment) is achieved by concurrently giving N-(phosphonacetyl)-L-aspartate (PALA), which leads to pyrimidine depletion-mediated death of non-transduced cells and cytosine, which rescues FCU1 containing cells via the pyrimidine salvage pathway. Negative selection (eradication) is induced once the prodrug 5-fluorocytosine is given. Murine NXS2 neuroblastoma cells transduced with FCU1 were effectively enriched in vitro, leading to a near-complete bystander effect. In vivo, enrichment-eradication led to decreased tumor growth and prolonged survival of mice bearing subcutanous NXS2 tumors. In conclusion, the enrichment-eradication approach can complement efforts to increase gene transfer efficacy, one major hurdle of cancer gene therapy.

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