ARE-regulated lentiviral HSV-TK/GCV gene therapy

Oxidative stress-regulated lentiviral TK/GCV gene therapy for lung cancer treatment

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Abstract

NRF2 is a transcription factor that regulates protection against a wide variety of toxic insults to cells, including cytotoxic cancer chemotherapeutic drugs. Many lung cancer cells harbor a mutation in either NRF2 or its inhibitor KEAP1 resulting in permanent activation of NRF2 and chemoresistance. In this study, we sought to examine whether this attribute could be exploited in cancer suicide gene therapy by using a lentiviral (LV) vector expressing herpes simplex virus thymidine kinase (HSV-TK/GCV) under the regulation of antioxidant response element (ARE), a cis-acting enhancer sequence that binds NRF2. In human lung adenocarcinoma cells where NRF2 is constitutively overexpressed, ARE activity was found to be high under basal conditions. In this setting, ARE-HSV-TK was more effective than a vector in which HSV-TK expression was driven by a constitutively active promoter. In a mouse xenograft model of lung cancer, suicide gene therapy with LV-ARE-TK/GCV was effective compared to LV-PGK-TK/GCV in reducing tumor size. We conclude that ARE-regulated HSV-TK/GCV therapy offers a promising approach for suicide cancer gene therapy in cells with high constitutive ARE activity, permitting a greater degree of therapeutic targeting to those cells.
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Introduction

Oxidative stress, an imbalance between the production and disposal of reactive oxygen species (ROS), plays an important role in carcinogenesis due to mutations in DNA, proteins and lipids caused by ROS (1). Importantly, many carcinogens act as reactive electrophilic intermediates. On the other hand, the effect of various anticancer drugs is based on ROS mediated apoptosis (2). Many cancers have developed resistance against anticancer drugs, and in drug-resistant cancer cells the expression of many self-defence genes such as antioxidant and phase II detoxifying enzymes as well as drug efflux proteins is increased (3-6).

The Kelch-like ECH-associated protein 1 (Keap1) – Nuclear factor erythroid-2 related factor 2 (Nrf2) pathway is the major sensor of endogenous and environmental oxidant and electrophile stress. Nrf2 is a member of the Cap’n’Collar basic leucine zipper (bZIP) family of transcription factors, and it recognizes the antioxidant response element (ARE) sequence in the regulatory regions of target genes. In basal conditions, Nrf2 is located in the cytoplasm and is repressed by the Keap1 dimer, which facilitates proteasomal degradation of Nrf2 through the Cullin3-Rbx1 ubiquitin ligase complex. On exposure to inducing agents, the interaction of Keap1 with Nrf2 is interrupted so that Keap1 no longer is able to direct Nrf2 to proteasomal degradation, and therefore Nrf2 stabilizes and translocates to the nucleus. In the nucleus, Nrf2 binds to ARE as a heterodimer with small Maf proteins, and drives the expression of large number of ARE-regulated genes (7, 8). These genes encode e.g. antioxidant and phase II detoxification genes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase (GCL), and heme oxygenase-1 (HO-1) (9). Nrf2 has also been shown to regulate the expression of some efflux proteins, such as multidrug resistance proteins (10, 11).
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Nrf2 has long been recognized as the key mediator of cancer chemoprevention (12, 13). Several chemopreventive agents such as dithiolethiones and isothiocyanates have been shown to exert their actions via Nrf2 activation, and some of these agents such as oltipraz and sulforaphane have entered clinical trials. In addition, Nrf2-deficient mice are more susceptible to chemical-induced carcinogenesis (7, 14). However, it has been recently discovered that Nrf2 not only protects against cancer development, but can also protect cancer cells against chemotherapeutic agents thereby providing advantage for cancer growth (15). Of note, it has been reported that Nrf2 is overexpressed in several lung cancer cell lines and patient samples, and this is mainly due to mutations in Nrf2 or Keap1. This has led to a notion that enhanced ARE activity is in part responsible for the development of chemoresistance, and that inhibition of the Nrf2 signaling pathway could be beneficial in certain cancers (16-19).

Suicide gene therapy is widely used in cancer gene therapy. One of the most common suicide gene therapy systems is based on thymidine kinase (TK) gene of the herpes simplex virus. When TK is transferred into cancer cells and cells are treated with a pro-drug ganciclovir (GCV), GCV is metabolized to GCV-monophosphate. Monophosphorylated GCV is further phosphorylated by cellular kinases into a triphosphate form, which is toxic to cells, leading to death of not only tumor cells containing transferred TK gene, but also the neighboring cells due to the bystander effect (20). To enhance the tumor selectivity of the system, several natural or synthetic enhancer-promoters have been used to drive TK expression. For example, systems inducible by hypoxia, radiation and oxidative stress have been developed and successfully applied to tumor xenograft models (21-23).

The notion that ARE activity is constitutively high in certain cancer cell types prompted us to study whether this attribute could be exploited in HSV-TK/GCV gene therapy by
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using the ARE element to drive the transgene expression. Here we show that ARE-regulated HSV-TK/GCV therapy is effective both in vitro and in vivo in A549 lung cancer cells, which have high constitutive ARE activity due to mutations in Keap1 protein.
Materials and methods

Cell culture

All the cell lines used were purchased from The American Type Culture Collection between years 2001 and 2010 and were systematically validated for the expression of specific marker genes, such as Nrf2 and Keap1. Human lung adenocarcinoma epithelial A549 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10 % (v/v) FBS (Sigma) and 1 % penicillin/streptomycin (Sigma). Human bronchiolar pulmonary adenocarcinoma NCI-H441 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10 % FBS, 1 % sodium-pyruvate (Sigma), 1 % HEPES (Sigma) and 1 % penicillin/streptomycin. Bronchiolar Beas2B cells were maintained in Bronchial Epithelial Cell Growth Medium (Lonza) and were cultured in precoated (0.01 mg/ml fibronectin (Sigma), 0.03 mg/ml bovine collagen type I (Cultrex) and 0.01 mg/ml bovine serum albumin (Sigma) in HBSS (Sigma)) plates.

DNA sample collection and sequencing

Beas2B and NCI-H441 cells were lysed to nuclear extraction buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.1% NP-40) and centrifuged for 5 min, 1500 x g, at +4°C to collect the nuclei. The nuclei were lysed to SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and the samples were sonicated for 5 min (30s-30s cycles) using Bioruptor UCD-200...
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instrument (Diagenode) and centrifuged full speed for 10 min at +4°C. DNA was extracted using a DNA extraction kit (JETquick, GENOMED) according to the kit protocol. All coding exons for KEAP1 and exon2 for Nrf2 were amplified by PCR using Phusion High-Fidelity DNA Polymerase (M0530S, New England Bio Labs) and appropriate primers from two recent publications (18, 24). Primers were synthesized by TAG Copenhagen (Copenhagen, Denmark). PCR products were purified and analyzed by sequencing at the DNA Sequencing Facility of the A. I. Virtanen Institute, University of Eastern Finland.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted with TRI Reagent (Sigma-Aldrich). 1 μg of the RNA was used for cDNA synthesis by High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems). The relative expression levels were measured by quantitative real-time PCR using relative standard curve method (StepOnePlus™ Real-Time PCR systems, Applied Biosystems) with specific Assays-on-Demand target mixtures for Nrf2 (NFE2L2, Hs00232352_m1, Applied Biosystems) and KEAP1 (Hs00202227_m1, Applied Biosystems).

**Western blotting**

Cells were lysed and protein concentrations were measured with the BCA kit (Pierce). Equal amounts of protein were used for electrophoresis. Proteins were transferred to nitrocellulose membrane, blocked o/n in +4°C with 5% non-fat dry milk in TBS-Tween, and incubated for 2 hours in room temperature or o/n in +4°C with the primary rabbit polyclonal Nrf2 antibody (sc-
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13032, Santa Cruz Biotechnology), with the rabbit polyclonal Keap1 antibody (cat. # 1050, Proteintech Europe) or with the rabbit polyclonal β-actin antibody (#4967L, Cell Signaling Technology). Blots were detected by incubating the blots with HRP-conjugated secondary antibody (Thermo Scientific) and by using ECL Plus Western blotting Detection System (GE Healthcare) with Typhoon 9400 (GE Healthcare). Images were processed with ImageQuant™ TL (GE Healthcare).

Lentiviral constructs

The cloning of third generation lentiviral vectors which express the luciferase reporter gene under minimal SV-40 promoter (LV-control) or minimal SV-40 promoter together with four AREs from human glutamate-cysteine ligase modifier subunit (LV-ARE-luc) have been described elsewhere (25). Lentiviral ARE-TK (LV-ARE-TK) was cloned similarly but the luciferase gene was replaced with HSV-TK (Fig. 3A). For cloning TK under constitutively active human phosphoglycerate kinase (hPGK) promoter expressing lentiviral vector (LV-PGK-TK), thymidine kinase (TK) -gene from pUC-TGL-TK (26) was cloned to pENTR-221 (Invitrogen) and further from this plasmid to the lentiviral backbone using Gateway® Technology (Invitrogen). The lentiviral vector containing no transgene was used as a control vector (empty virus) in MTT assays and in vivo (25).

Third generation lentiviruses were produced (27) and the titer was assessed by HIV p24 ELISA (Perkin Elmer). To test the transduction efficiency, the construct expressing GFP under hPGK promoter was used (28).
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**Luciferase assays**

For the luciferase assay, 20 000 to 30 000 cells were plated on black 96-well view plates (Perkin Elmer). The transduction efficiency was assessed by transducing cells with GFP expressing lentivirus and FACS analysis as in (28). Multiplicities of infection (MOI) that yielded similar number of GFP positive cells in FACS analysis (~85%) were used for transduction. For Beas2B and A549 cells MOI 10 and for NCI-H441 MOI 20 were used to transduce with LV-control or LV-ARE-luc constructs. 48 hours later cells were treated with 50-200 µM diethylmaleate (DEM, Sigma), 50-100 µM tert-butylhydroquinone (tBHQ, Sigma), 100 µM ganciclovir (GCV, Roche) or 100-500 nM doxorubicin (Sigma) and 16 hours later luciferase assay was performed with Britelite Reporter Assay System (Perkin Elmer) according to the manufacturers’ instructions. Results were normalized to protein measured with the BCA-kit. All data is depicted relative to luciferase activity of LV-control.

**Lentiviral HSV-TK/GCV therapy in vitro**

20 000 to 30 000 cells were plated on 96-well plates, and cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK. The next day, medium was changed and 24 hours later cells were treated with 10-100 µM GCV. GCV treatment was repeated two days later. 5 days after the first GCV treatment the cell survival was measured using CellTiter 96® Aqueous One Solution Cell Proliferation-assay (Promega) according to the manufacturers’ instructions. When DEM was used in experiments, it was given 24 hours after transduction in 50 µM concentration together with GCV and was repeated with the GCV treatment. Similarly, doxorubicin was given 24 hours...
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after the first GCV treatment using 1-500 nM concentration, and again together with GCV 48 h later.

**Lentiviral HSV-TK/GCV therapy in vivo**

A549 cells were seeded on 10 cm plates and transduced with empty virus, LV-ARE-TK or LV-PGK-TK using MOI 10 and grown until near confluent. Cells were washed once with PBS (Sigma), trypsinized (Sigma) and pelleted by centrifugation. Cell pellets were washed with Optimem (Gibco) and finally suspended in a small volume of Optimem. 3 million cells were injected in 50 µl volume subcutaneously into the flanks of 7 weeks old male NMRI nu/nu mice (Charles River, Germany). Two tumors were injected per mouse. After one week, when the tumor sizes were approximately 15-25 mm³, mice were given GCV (Roche) 50 mg/kg/day *i.p.* twice a day for two weeks. Control groups were injected with 0.9% NaCl. Tumor sizes were measured twice a week, and tumor volumes were calculated as $4/3*\pi*(a/2*b/2*c/2)$ where a, b and c are tumor width, length and height, respectively. After GCV treatment mice were followed for additional 7 days, and were then sacrificed. All mice were kept in the National Laboratory Animal Centre (Kuopio, Finland), and the experimental procedure was approved by the National Animal Experiment Board (Finland).

**Statistical analysis**

Statistical analyses were performed using Graph Pad Prism Software. (GraphPad Software, Inc.). Statistical significance of *in vitro* data was evaluated by paired t-test and for *in vivo* data 2way
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ANOVA posthoc was used. P<0.05 was considered statistically significant. Results are expressed as mean±SEM.
Results

Status of KEAP1 and NRF2 in Beas2B, NCI-H441 and A549 cells

Recently, it has been shown that the transcription factor Nrf2 is overexpressed in several lung cancer cell lines (16-19). In A549 cells, a Gly→Cys amino acid change at the first Kelch domain of KEAP1 (amino acid position 333) has been identified (18), inhibiting Keap1 function and rendering Nrf2 overactive. The sequence of NRF2 has been shown to be wild type in A549 cells (24). The KEAP1 gene in Beas2B has been found to be wild type (18), but the sequence of Nrf2 in these cells and both Nrf2 and Keap1 in NCI-H441 are not known. All identified cancer mutations for NRF2 are clustered in exon 2 in sites that are critical for the binding of Nrf2 to KEAP1 (29). Therefore only exon 2 was sequenced in Beas2B and NCI-H441 cells and was found to be wild type in both cell lines. The locations for KEAP1 mutations are more evenly distributed throughout the gene, and the whole KEAP1 gene was sequenced in NCI-H441. In NCI-H441, KEAP1 exon 4 contained a synonymous substitution in codon 471, which had no impact on the amino acid sequence (CTC (Leu) → CTG (Leu)).

Keap1 expression has been suggested to be regulated via epigenetic mechanisms in human lung cancer cells and tissues (30, 31). We therefore also assessed the mRNA and protein expression of both Nrf2 and Keap1 in all three cell lines (Fig. 1A-E). There were no statistically significant differences in Nrf2 mRNA expression between different cell lines (Fig. 1A), whereas Keap1 mRNA was significantly lower in NCI-H441 cells in comparison to Beas2B and A549 cells (Fig. 1B). In line with previous reports (18, 32), Nrf2 protein is increased in comparison to
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Beas2B and NCI-H441 cells (Fig. 1C and E), whereas the lowest Keap1 protein levels were found in A549 cells (Fig. 1D and F).

**Basal and inducible ARE activities in Beas2B, NCI-H441, and A549 cells**

We then sought to evaluate the basal ARE activities in Beas2B, A549 and NCI-H441, by transducing cells with the LV-control or with the LV-ARE-luc reporter construct (Fig. 2A) (25). A549 cell line had the highest ARE activity, 26-fold higher than the LV-control, whereas NCI-H441 cell line had the lowest activity having only 2-fold increase in luciferase activity in comparison to LV-control (Fig. 2B). The Beas2B control cells had 5-fold ARE activity compared to LV-control.

Next we examined whether it would be possible to further increase the ARE activity in A549, NCI-H441 and Beas2B cells by using Nrf2 activators tBHQ and DEM. The inducible ARE activity was measured by transducing cells with either LV-control or LV-ARE-luc reporter construct, and by treating the cells with different concentrations of DEM and tBHQ (Fig. 2C-E). In A549 cells, in which the basal ARE activity was very high, it was not increased by Nrf2 activators any further (Fig. 2E). In contrast, in both Beas2B and NCI-H441 cells, in which the ARE activity was low in basal conditions, the activity was increased by both DEM and tBHQ (Fig. 2C and D).

We also examined the effect of ganciclovir (GCV) and doxorubicin, which were used in subsequent experiments, on ARE activity in A549 and NCI-H441 cells. It was found that neither GCV nor doxorubicin had an effect on ARE activity in either cell line (Supplemental Fig. S1).
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ARE-regulated HSV-TK/GCV therapy is effective in A549 and NCI-H441 cell lines in vitro

Inasmuch as in A549 cells the constitutive ARE activity was high and in NCI-H441 cells the ARE activity was highly inducible by Nrf2 activators, we next assessed whether ARE-regulated vectors could be used to target suicide gene therapy into lung cancer cells. In order to study this, we used an established pro-drug activating HSV-TK/GCV gene therapy approach that has been successfully used to treat non-small lung cancer in vitro and in vivo (33, 34). To this end, we cloned thymidine kinase (TK) into LV-ARE-luc by displacing luciferase reporter gene with the HSV-TK gene (Fig. 3A), and the vector was used to study the effect of ARE-regulated HSV-TK/GCV therapy in vitro in A549 and in NCI-H441 cell lines. Cells were transduced with an empty lentivirus containing no transgene (empty virus) or LV-ARE-TK. Two days after transduction cells were incubated with different concentrations of GCV for 5 days, and the cell survival was measured with the MTT-assay. There was a concentration dependent reduction of viability in LV-ARE-TK transduced A549 cells (Fig. 3B). With 100 µM GCV, less than 10% of cells were viable at the end of the study. GCV treatment did not affect the viability of non-transduced cells or cells transduced with empty virus (Fig. 3B).

In NCI-H441 cells, on the other hand, in which the basal ARE activity is lower, LV-ARE-TK/GCV therapy was not as effective as in A549 cells. Even after 100 µM GCV treatment almost 40% of cells were still viable (Fig. 3C).

ARE-regulated HSV-TK/GCV suicide therapy is more efficient than constitutive HSV-TK/GCV therapy in A549 cells but not in NCI-H441 cells in vitro
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To compare the efficacy of ARE-regulated HSV-TK/GCV therapy with constitutive HSV-TK/GCV therapy, A549 cells or NCI-H441 cells were transduced either with LV-ARE-TK or with lentiviral vector expressing HSV-TK under the constitutively active human phosphoglycerate kinase (hPGK) promoter and treated as previously described. In A549 cells, LV-ARE-TK was equally effective with low GCV concentrations and significantly more effective with higher concentrations than LV-PGK-TK (Fig. 3D).

In contrast, constitutive LV-PGK-TK/GCV therapy was significantly more effective than ARE-regulated TK/GCV therapy with all GCV concentrations used in NCI-H441 cells (Fig. 3E).

**The combination of LV-ARE-TK/GCV therapy with DEM in NCI-H441 cells or doxorubicin in A549 cells enhanced the effect of LV-ARE-TK/GCV therapy**

Since in NCI-H441 cells the ARE activity was highly inducible with DEM and tBHQ, we next examined whether 50 µM DEM could be used to increase the effect of ARE-regulated HSV-TK/GCV therapy. There was a small but significant (with 10 µM GCV p=0.0451, with 50 µM GCV p=0.0197) increase in cytotoxicity when 10 µM or 50 µM GCV was used together with 50 µM DEM compared to GCV treatment alone, but no further effect was seen when DEM was used together with 100 µM GCV (Fig. 4A).

In addition, we evaluated the efficacy of doxorubicin chemotherapy combined with ARE-regulated TK/GCV suicide therapy in A549 cells (Fig. 4B). Although doxorubicin has been shown to increase cellular ROS formation (35), it did not increase the ARE activity, assessed by the luciferase assay (Supplemental Fig. S1C). Doxorubicin at concentrations 250 nM and 500
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nM, that alone were not toxic to the cells, significantly enhanced the efficacy of HSV-TK/GCV therapy.

The effect of ARE-regulated HSV-TK/GCV therapy in vivo

Because ARE-regulated HSV-TK/GCV suicide gene therapy was efficient in A549 cells in vitro, we next examined its efficacy in vivo using subcutaneous tumor model in NMRI nu/nu mice. A549 cells were transduced with empty virus, LV-PGK-TK or LV-ARE-TK, collected and injected subcutaneously into the flanks of nude NMRI nu/nu mice, two tumors per mouse. After one week, when tumors were approximately 20 mm³, GCV was injected i.p. twice a day 50 mg/kg/day for two weeks. Mice were followed for another week and then sacrificed. In both control groups (nontransduced cells and empty virus transduced cells) tumors grew faster than in other groups in which TK was expressed (Fig. 5A and B). Interestingly, LV-ARE-TK alone without GCV caused a significant reduction of tumor growth in comparison to LV-PGK-TK transduced tumors (Fig. 5B), presumably because of the high expression of transgene that occupies the transcriptional and translational machinery thus causing cytostasis. However, the tumors were statistically significantly smaller in size in both LV-PGK-TK and LV-ARE-TK in mice receiving GCV in comparison to respective saline controls from day 17 onwards, and by day 28 there were no visible tumors left. In all other groups there were clearly visible tumors at the end of the study.
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Discussion

Accumulating evidence suggests that high constitutive expression of Nrf2 and subsequent upregulation of phase II enzymes and other pro-survival genes plays a pivotal role in cancer chemoresistance (36). The growing list of cancer types in which the Keap1-Nrf2-system has been shown to be hyperactive includes lung (24), gall bladder (37), pancreatic (38), hepatocellular (39), ovarian (40) and prostate (41) cancers. This is either due to mutations in Keap1 or Nrf2, accumulation of proteins that disrupt the Keap1-Nrf2 interaction such as p62, or Keap1 promoter methylation resulting in reduced Keap1 expression (reviewed in (29). In A549 lung cancer cells, both Keap1 promoter hypermethylation (30, 42) and a point mutation in the Nrf2 interacting domain in Keap1 (43) have been found. In this study, we sought to examine whether dysregulation of the Keap1-Nrf2-system could be exploited to therapeutic advantage by utilizing the Nrf2-driven ARE-element to drive the expression of the HSV-TK suicide gene. The data presented herein demonstrates the feasibility and efficacy of such approach in cancer gene therapy.

Ideally, cancer gene therapy should affect only the cancerous tissue having minimal or no effect on healthy, dividing cells. Transcriptional targeting is one of the approaches in which the selectivity is improved by using promoter elements that are primarily active in cancer cells. With respect to gene therapy using the HSV-TK/GCV suicide gene approach, tumor microenvironment-specific promoters that have been used for this purpose include hypoxia response elements derived from the VEGF gene (44) and the glucose responsive hexokinase II promoter (45). In addition, redox-responsive DNA motifs have been used to regulate the HSV-TK/GCV suicide gene therapy in cancer cells that have higher ROS levels than healthy cells.
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(23). ROS are produced also in normally dividing cells as a by-product of cellular metabolism, but their production as well as the activity of ROS-responsive promoters is increased in certain malignant cells, by two- to three-fold in comparison to healthy cells assessed by the luciferase reporter assay (23). Similarly, Nrf2 is broadly expressed also in healthy cells, but dysregulation of its activity results in a substantially higher basal activity in A549 cells in comparison to Beas2B and NCI-H441 cells (Fig. 1B), sufficient to confer specificity to this treatment. Remarkably, ARE-driven HSV-TK was more effective than constitutively active PGK promoter in reducing viability in HSV-TK/GCV gene therapy (Fig. 3D) in A549 cells in vitro, demonstrating the efficacy of this approach. In vivo, both PGK-TK and ARE-TK were equally effective, as the response to treatment was complete in both groups treated with GCV, with complete disappearance of tumors in both groups by day 28.

In addition to the use of ARE in the regulation of transgene expression in cells with high basal ARE activity, ARE-driven vectors could be potentially used as an inducible vector system regulated by Nrf2 activators. With respect to cancer, it is interesting to note that certain anticancer agents (46) and ionizing radiation (47) cause a modest but measureable increase in ARE activity, thus providing a possibility that chemotherapeutic agents or radiotherapy could also be used to boost the efficacy of ARE-HSV-TK/GCV therapy. However, in NCI-H441 cells in which the basal ARE activity is low but highly inducible by commonly used ARE-activators DEM and tBHQ, as assessed by the luciferase reporter assay (Fig. 2C), DEM treatment did not substantially enhance toxicity of the ARE-HSV-TK/GCV therapy (Fig. 4A). Although it is possible that factors such as the selection of the inducing agent and timing of administration were not optimal to maximize the efficacy of ARE-driven HSV-TK expression, it is unlikely that the inducible ARE activation could be effectively used in vivo. However, doxorubicin, which on
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its own had no impact on ARE activity in our setting (Supplementary Fig. S1C and D) nor effect
on cell viability in control cells (Fig. 4B), substantially enhanced the cytotoxic effect of ARE-
HSV-TK (Fig. 4B). As single agent cancer therapies often result in drug resistance and are
therefore less likely to be curative than combination strategies targeting multiple pathways (48,
49), the synergistic effect of the combination of doxorubicin with the ARE-HSV-TK is therefore
encouraging and suggests that this combination could be used to enhance therapeutic efficacy.

In conclusion, we have shown that ARE-regulated HSV-TK/GCV therapy is a
powerful option for cancer treatment in cells with high constitutive Nrf2 expression.
Furthermore, transcriptional targeting with the ARE element, either alone or in combination with
other targeting methods, may improve the safety of HSV-TK/GCV therapy.

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Figure Legends

**Figure 1. Nrf2 and Keap1 status in Beas2B, NCI-H441 and A549 cells.** A and B) Relative Nrf2 and Keap1 mRNA levels in Beas2B, NCI-H441 and A549 cells. Equal amount of total RNA was used in quantitative RT-PCR. Data is depicted as relative to Nrf2 or Keap1 mRNA in Beas2B cells. C and E) Basal Nrf2 and Keap1 protein levels were measured by Western blotting. Equal amount of protein was used in each well and anti-β-actin was used as a loading control. D and F) Densitometric analyses of Nrf2 and Keap1 immunoblots relative to β-actin. Data is depicted relative to Beas2B cells. * p<0.05

**Figure 2. Basal and inducible ARE activities in Beas2B, NCI-H441 and A549 cells.**

A) Structures of the lentiviral ARE luciferase reporter (LV-ARE-luc) and control vector (LV-control) used to study ARE activity. The ARE construct contains four AREs from human glutamate-cysteine ligase modifier subunit, minimal SV40 promoter and luciferase reporter gene, whereas the control has the minimal SV40 promoter and luciferase reporter gene only. B) Basal ARE activities in each cell line were measured by transducing cells with LV-control or LV-ARE-luc vectors, and luciferase activities were measured 48 hours later. Results were normalized to protein and are presented as fold increase in luciferase activity in LV-ARE-luc vs. LV-control, n= 4, mean +/- SEM. C-E) For measuring inducible ARE-activities Beas2B (C), NCI-H441 (D) and A549 (E) cells were transduced with LV-control or LV-ARE-luc vectors. DEM or tBHQ were added to the culture medium 24 hours after transduction. Luciferase activities were measured the next day and were normalized to proteins. The data is depicted
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relative to luciferase activity of non-treated cells transduced with LV-control, mean +/- SEM, n=4-6.

**Figure 3. The effect of ARE-regulated HSV-TK/GCV suicide gene therapy.** A) A lentiviral vector containing HSV-TK gene, four AREs and minimal SV40 promoter was cloned. HSV-TK regulated by a constitutively active hPGK promoter was also used in this study. B and C) A549 and NCI-H441 cells were transduced with empty lentiviral vector containing no transgene (empty virus) or LV-ARE-TK vector using MOI 10. After 48 hours GCV was added to cell culture medium and cells were incubated in the presence of GCV for five days. Cell viability was measured with the MTT assay and is depicted percent viability vs. no virus control without GCV, mean +/- SEM, n=12-18. D and E) A549 and NCI-H441 cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK vector using MOI 10. After 48 hours, cells were treated with GCV and 5 days later cell viability was measured using the MTT assay. The data is depicted relative to non-transduced cells without GCV, mean +/-SEM, n=12-18. * p<0.05; ** p<0.01; ***p<0.001.

**Figure 4. Enhancement of the effect of ARE-regulated HSV-TK/GCV suicide gene therapy by DEM and doxorubicin.** A) NCI-H441 cells were transduced with empty virus or LV-ARE-TK vector using MOI 10. 48 hours later cells were treated with GCV and the next day 50 µM DEM was added to cell culture medium. After 5-day GCV treatment cell viability was measured using the MTT assay. B) A549 cells were transduced with empty virus or LV-ARE-TK vector using MOI 10. 48 hours after transduction cells were treated with GCV (25 µM) following the addition of doxorubicin (doxo) treatment the next day. Doxo/GCV treatment was renewed every
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other day. Cell viability was measured with MTT assay 5 days after the beginning of GCV treatment. The data is depicted relative to non-transduced cells without GCV, mean +/-SEM, n=6-8. * p<0.05; ** p<0.01; ***p<0.001.

Figure 5. The effect of ARE-regulated HSV-TK/GCV suicide gene therapy in vivo. A549 cells were transduced with empty virus, LV-ARE-TK or LV-PGK-TK using MOI 10. Cells were collected when near confluent and 3 million cells were injected subcutaneously into 38 NMRI nu/nu mice (two tumors per mouse). The groups were as follows: no virus (6 mice, n=12), empty virus (6 mice, n=12), LV-PGK-TK virus without GCV (8 mice, n=16), LV-PGK-TK virus + GCV (50 mg/kg/day) (6 mice, n=12), LV-ARE-TK virus without GCV (6 mice, n=12) and LV-ARE-TK + GCV (50 mg/kg/day) (6 mice, n=12). A week after tumor implantation GCV (or saline) was given i.p. twice a day for 14 days, and a week later mice were sacrificed. Tumor sizes were measured twice a week and tumor volumes were calculated using the following formula:

tumor volume = \frac{4}{3} \pi \left(\frac{a}{2}\right) \left(\frac{b}{2}\right) \left(\frac{c}{2}\right),

where a, b and c are tumor length, width and height. Results are presented as the tumor volume mm$^3$ +/- SEM * p<0.05; ** p<0.01; ***p<0.001 in comparison to non-treated LV-ARE-TK or LV-PGK-TK.
Leinonen et al. Fig. 1
Figure 4

Panel A:
- NCI-H441
- Percent cell viability
- No treatment
- 10 μM GCV
- 50 μM GCV
- 100 μM GCV
- 50 μM DEM
- Empty virus
- LV-ARE-TK

Panel B:
- A549
- Percent cell viability
- No treatment
- 10 nM doxo
- 100 nM doxo
- 250 nM doxo
- 500 nM doxo
- 25 μM GCV
- Empty virus
- LV-ARE-TK

Leinonen et al. Fig 4
A

no virus                                   empty virus
LV-PGK-TK -                              LV-PGK-TK + GCV
LV-ARE-TK -                              LV-ARE-TK + GCV

B

Leinonen et al Fig 5

Tumor volume (mm$^3$)

Day

LV-PGK-TK -
LV-ARE-TK -
LV-PGK-TK + GCV
LV-ARE-TK + GCV
...
Oxidative stress-regulated lentiviral TK/GCV gene therapy for lung cancer treatment

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