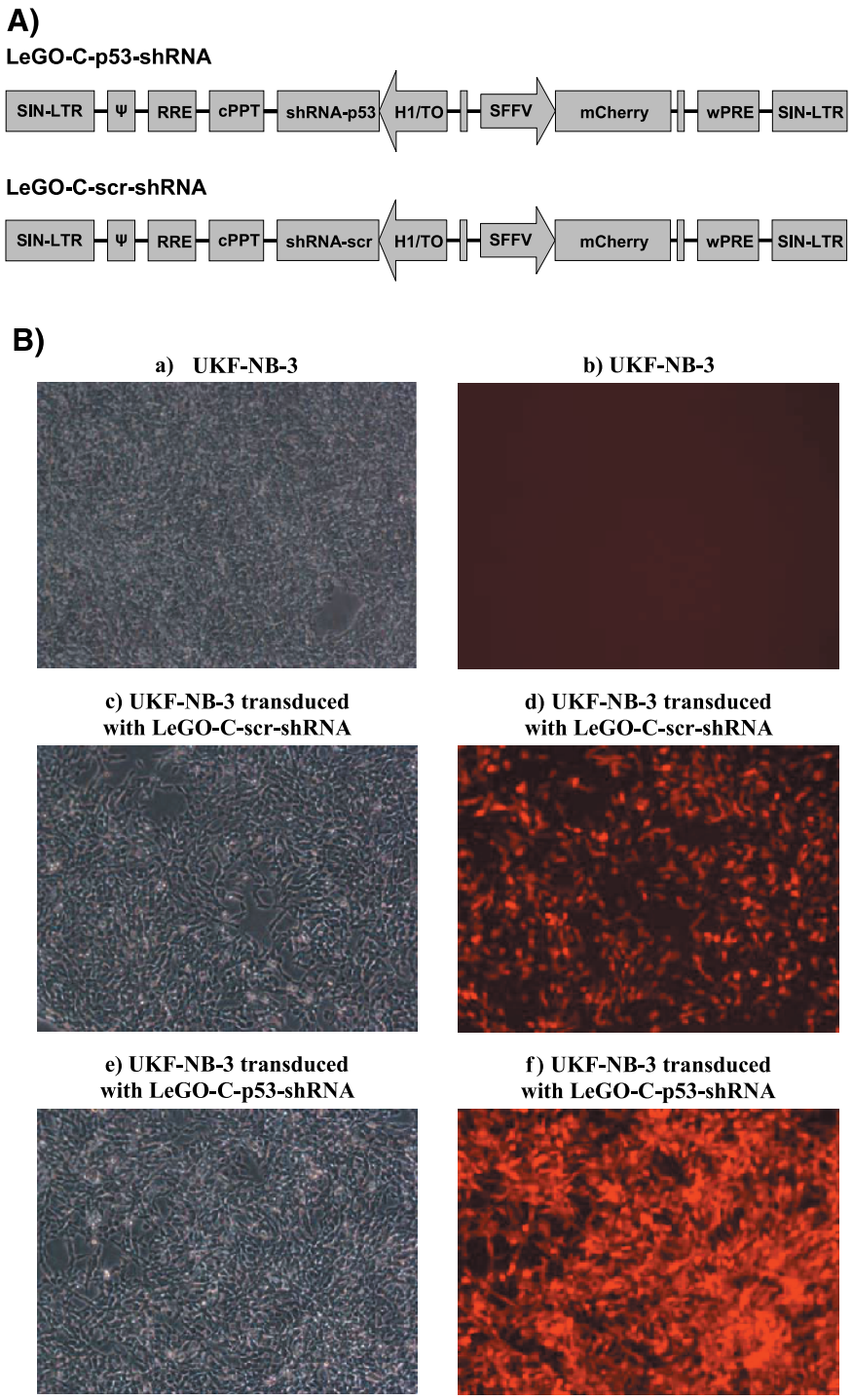
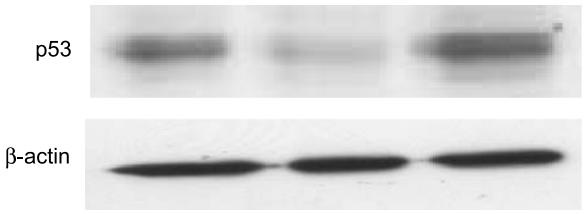


Figure W1. (A) Scheme of the lentiviral vectors, as integrated proviruses, that have been used in this study (not drawn to scale). Ψ indicates packaging signal; cPPT, central polypurine tract; H1/TO, RNA-polymerase III promoter; mCherry, a red fluorescent protein; RRE, rev-responsive element; SFFV, spleen focus–forming virus enhancer/promoter; shRNA-p53, short hairpin RNA against human p53; shRNA-scr, short hairpin RNA with no target (scrambled); SIN-LTR, self-inactivating long-terminal repeat; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. (B) UKF-NB-3, UKF-NB-3 transduced with LeGO-C-scr-shRNA (UKF-NB-3$^{scr\text{-}shRNA}$, and UKF-NB-3 transduced with LeGO-C-p53-shRNA (UKF-NB-3$^{p53\text{-}shRNA}$) cells were photographed by inverse light microscopy (a, c, and e) and by fluorescence microscopy (b, d, and f), using an IX71 fluorescence microscope (Olympus). Fluorescence dye mCherry was detected at 587/610 nm (excitation/emission). The nontransduced control cell line UKF-NB-3 showed no fluorescence (b). In contrast, UKF-NB-3$^{scr\text{-}shRNA}$ and UKF-NB-3$^{p53\text{-}shRNA}$ cells displayed high fluorescence due to successful transduction with shRNAs (d, f). (C) Western blot was carried out with UKF-NB-3 (a), UKF-NB-3$^{p53\text{-}shRNA}$ (b), and UKF-NB-3$^{scr\text{-}shRNA}$ (c) cells, detecting p53 (Alexis Biochemicals through AXXORA Deutschland) and β-actin as control (Sigma). The cell lines UKF-NB-3 and UKF-NB-3$^{scr\text{-}shRNA}$ showed clear expression of p53 (a and c). In contrast, UKF-NB-3$^{p53\text{-}shRNA}$ cells displayed significant decreased p53-expression (b) due to successful transduction with shRNA against p53. Densitometric analysis revealed at least 70% down-regulation of p53. (D) Concentrations of the MDM2 inhibitor nutlin-3a that non-genotoxically activates p53, its enantiomer nutlin-3b that shows 150-fold lower MDM2-inhibitory activity, and of the cytotoxic drugs vincristine and doxorubicin that reduce cell viability by 50% (IC_{50}) as indicated by MTT assay. Values are mean ± SD from three independent experiments. *$P < .05$ relative to nontransfected cells.

C)



D)

Cell line	IC_{50} nutlin-3a (μM)	IC_{50} nutlin-3b (μM)	IC_{50} vincristine (ng/ml)	IC_{50} doxorubicin (ng/ml)
UKF-NB-3	0.81 ± 0.15	> 20	0.25 ± 0.06	6.22 ± 1.97
UKF-NB-3$^{p53\text{-}shRNA}$	8.23 ± 1.73*	> 20*	1.10 ± 0.21*	19.98 ± 2.61*
UKF-NB-3$^{scr\text{-}shRNA}$	0.66 ± 0.15	> 20	0.31 ± 0.05	5.94 ± 0.89

Figure W1. (*continued*).

Materials and Methods

Cloning of Lentiviral Vectors

Standard molecular cloning techniques were used to generate viral vectors based on Lentiviral Gene Ontology (LeGO) vectors [1] (also http://www.LentiGO-Vectors.de). Maps and sequence data of the vectors are available on request. The 60-bp shRNA against human p53 or the 60-bp scrambled shRNA was cloned into LeGO-C using *Xba*I and *Xho*I. The internal promoter H1/TO transcribes the shRNA against p53 or the scrambled shRNA, respectively. The internal SFFV promoter drives the expression of the red fluorescent protein mCherry as a marker gene.

Generation of Viral Particles

Cell-free viral supernatants were generated by transient transfection of 293T packaging cells as described [2], using the third-generation packaging plasmids pMDLg/pRRE and pRSV-Rev [3] together with phCMV-VSV-G [2]. Supernatants containing pseudotyped vector particles were titrated on 293T target cells. Gene transfer rates were analyzed 2 days after transduction by fluorescence-activated cell sorting (FACS). Titers of 1.5×10^7 (LeGO-C-p53-shRNA) and 2×10^7 (LeGO-C-scr-shRNA) VSV-G pseudotyped virus particles per milliliter of unconcentrated supernatants were obtained.

Cell Culture and Lentiviral Gene Transfer

All cells were cultured in their respective growth medium supplemented with penicillin/streptomycin. For transduction of UKF-NB-3 target cells, 5×10^4 cells in 500 μl of medium were plated per well in a 24-well plate. The next day, 250 μl of viral supernatant per well was added in the presence of 8 μg/ml polybrene, and the plate was centrifuged at 1000*g* for 1 hour at room temperature. After another 3 hours in the cell culture incubator, medium was replaced.

Fluorescence Microscopy

Pictures were taken using an IX71 fluorescence microscope (Olympus, Hamburg, Germany). Fluorescence dye mCherry was detected at 587 nm emission/610 nm excitation.

Western Blot

Cells were lysed in Triton X sample buffer and separated by SDS-PAGE, as described before [4]. Proteins were detected using specific antibodies against β-actin (Sigma, Munich, Germany) or p53 (Alexis Biochemicals through AXXORA Deutschland, Lörrach, Germany) and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham through GE Healthcare, Munich, Germany).

MTT Assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 96 hours of incubation modified as described before [5].

References

[1] Weber K, Bartsch U, Stocking C, and Fehse B (2008). A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol Ther* **16**, 698–706.

[2] Beyer WR, Westphal M, Ostertag W, and von Laer D (2002). Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range. *J Virol* **76**, 1488–1495.

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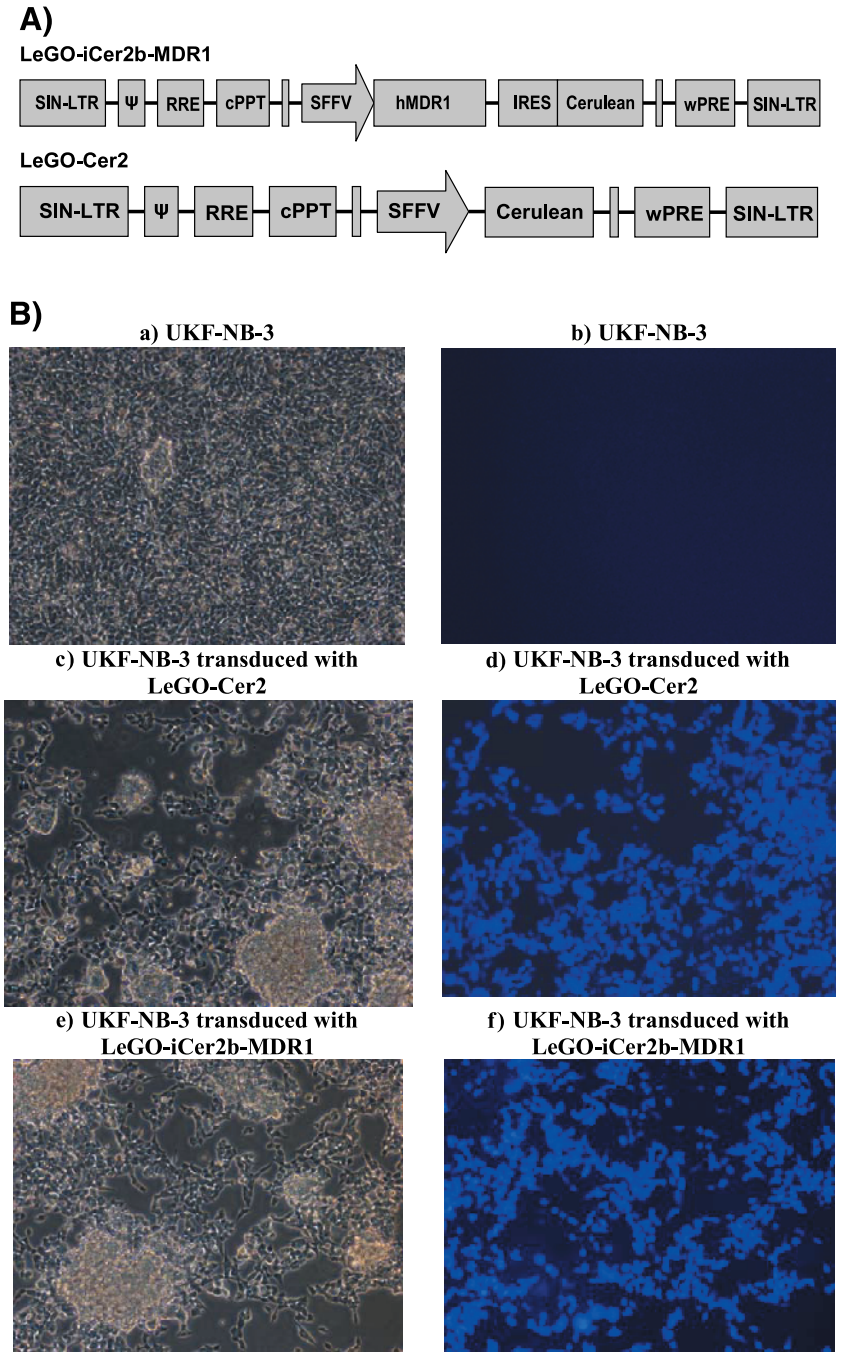
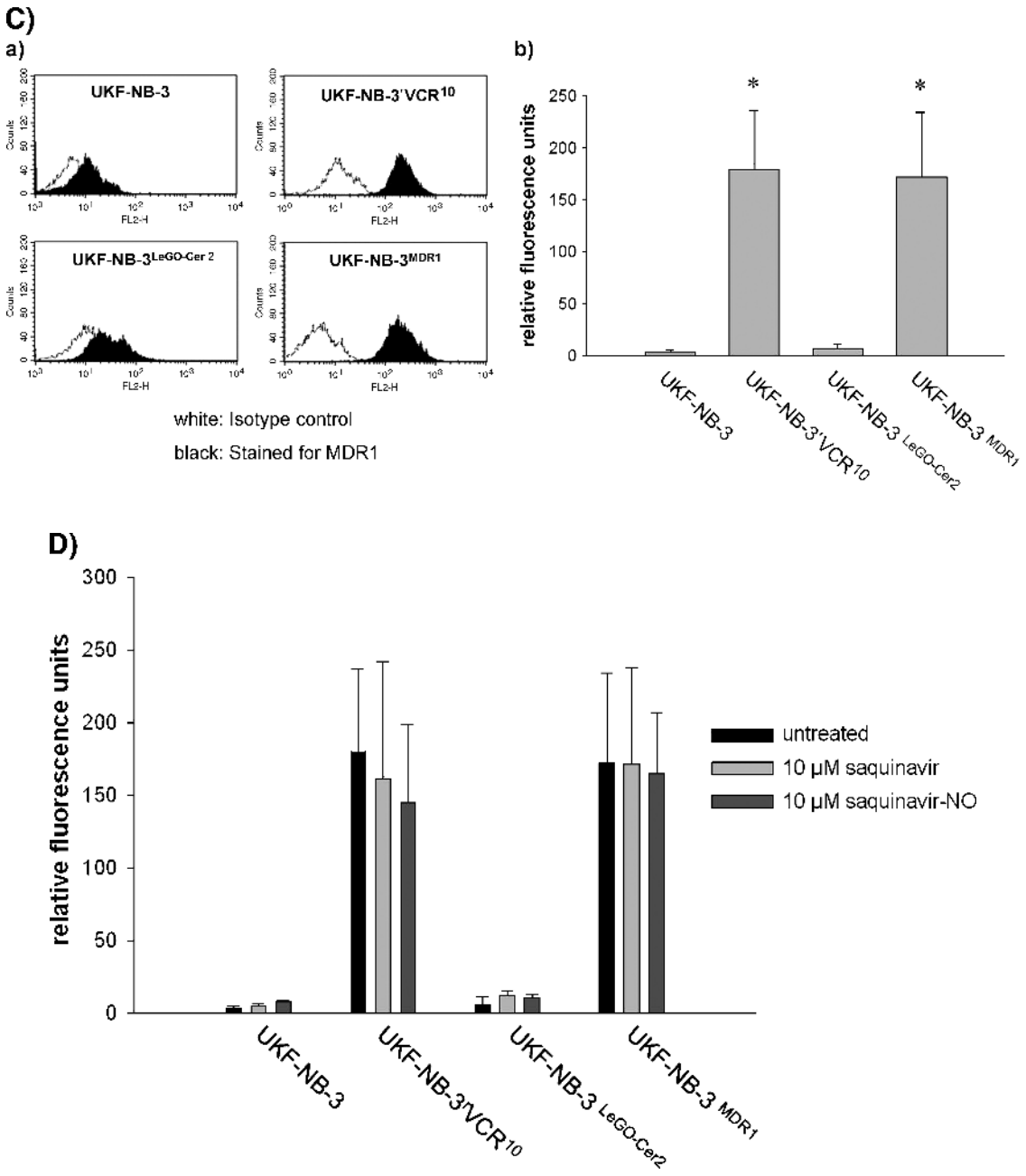


Figure W2. (A) Scheme of the lentiviral vectors, as integrated proviruses, that have been used in this study (not drawn to scale). Ψ indicates packaging signal; Cerulean, a blue variant of the enhanced green fluorescent protein; cPPT, central polypurine tract; hMDR1, human multiple drug resistance gene cDNA; IRES, internal ribosome entry site of the EMCV; RRE, rev-responsive element; SFFV, spleen focus–forming virus enhancer/promoter; SIN-LTR, self-inactivating long terminal repeat; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. (B) UKF-NB-3, UKF-NB-3 transduced with LeGO-Cer2 (UKF-NB-$3^{LeGO\text{-}Cer2}$), and UKF-NB-3 transduced with LeGO-iCer2-MDR1 (UKF-NB-3^{MDR1}) cells were photographed by inverse light microscopy (a, c, e) and by fluorescence microscopy (b, d, f), using an IX71 fluorescence microscope (Olympus). Fluorescence dye Cerulean was detected at 433/475 nm (excitation/emission). The nontransduced control cell line UKF-NB-3 showed no fluorescence (b). In contrast, UKF-NB-$3^{LeGO\text{-}Cer2}$ and UKF-NB-3^{MDR1} cells displayed high fluorescence due to successful transduction with LeGO-vectors (d, f). (C) Flow cytometric analysis of MDR1 expression. (a) Flow cytometric histograms of the cell lines UKF-NB-3 (negative control), UKF-NB-3^{r}VCR^{10} (positive control), UKF-NB-3 transduced with 100 μl of control vector LeGO-Cer2 (UKF-NB-$3^{LeGO\text{-}Cer2}$) and UKF-NB-3 transduced with 100 μl of human MDR1-expressing vector LeGO-iCer2b-MDR1 (UKF-NB-3^{MDR1}). (b) Quantitative analysis of flow cytometry data. *$P < .05$ relative to nontransduced UKF-NB-3 cells. (D) Flow cytometric analysis of MDR1 expression in the cell lines UKF-NB-3, UKF-NB-3^{r}VCR^{10}, UKF-NB-$3^{LeGO\text{-}Cer2}$, and UKF-NB-3^{MDR1} untreated (black bars), treated with 10 μM saquinavir for 5 days (gray bars) or treated with 10 μM saquinavir-NO for 5 days (dark gray bars). Incubation with saquinavir or saquinavir-NO had no influence on MDR1 expression. (E) Concentrations that decrease cell viability by 50% (IC_{50}) for the MDR1 substrate vincristine investigated by MTT assay (values are means ± SD). MDR1-expressing cell lines UKF-NB-3^{MDR1} and UKF-NB-3^{r}VCR^{10} showed higher IC_{50} values for vincristine compared with MDR1-negative cell lines UKF-NB-3 and UKF-NB-$3^{LeGO\text{-}Cer2}$. $^{1}P < .05$ relative to UKF-NB-3. $^{2}P < .05$ relative to UKF-NB-3^{r}VCR^{10}. $^{3}P < .05$ relative to UKF-NB-3^{MDR1}.



E)

Cell line	IC_{50} vincristine (ng/ml)	IC_{50} vincristine (ng/ml) + verapamil (10µM)
UKF-NB-3	0.20 ± 0.08	0.15 ± 0.07
UKF-NB-3'VCR^{10}	40.29 ± 5.07[1]	1.16 ± 0.21[2]
UKF-NB-3$^{LeGO-Cer2}$	0.28 ± 0.02	0.32 ± 0.09
UKF-NB-3^{MDR1}	13.17 ± 1.35[1]	0.13 ± 0.06[3]

Figure W2. (*continued*).

Materials and Methods

Cloning of Lentiviral Vectors

Standard molecular cloning techniques were used to generate viral vectors based on Lentiviral Gene Ontology (LeGO) vectors [1] (also http://www.LentiGO-Vectors.de). Maps and sequence data of the vectors are available on request. In the first step, the multiple cloning site of the lentiviral vector LeGO-Cer2 was inverted, resulting in LeGO-iCer2b. Then, the 3842-bp cDNA of the human *MDR1* gene was taken out of the γ-retroviral vector SF91m3.IRES.GFP [2,3] (kindly provided by A. Schambach and C. Baum, MH Hannover, Germany) and cloned into LeGO-iCer2b using *Bam*HI and *Not*I. The internal SFFV promoter of this vector transcribes a bicistronic messenger RNA (mRNA) with an internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV), expressing the hMDR1 together with the blue fluorescent protein Cerulean as a marker. The vector LeGO-Cer2 only expressing Cerulean served as a control.

Generation of Viral Particles

Cell-free viral supernatants were generated by transient transfection of 293T packaging cells as described [4], using the third-generation packaging plasmids pMDLg/pRRE and pRSV-Rev [5] together with phCMV-VSV-G [4]. Supernatants containing pseudotyped vector particles were titrated on 293T target cells. Gene transfer rates were analyzed 2 days after transduction by fluorescence-activated cell sorting (FACS). Titers of 4×10^6 (LeGO-iCer2b-MDR1) and 8×10^6 (LeGO-Cer2) VSV-G pseudotyped virus particles per milliliter of unconcentrated supernatants were obtained.

Cell Culture and Lentiviral Gene Transfer

All cells were cultured in their respective growth medium supplemented with penicillin/streptomycin. For transduction of UKF-NB-3 target cells, 5×10^4 cells in 500 μl of medium were plated per well in a 24-well plate. The next day, 250 μl of viral supernatant per well was added in the presence of 8 μg/ml polybrene, and the plate was centrifuged at 1000*g* for 1 hour at room temperature. After another 3 hours in the cell culture incubator, medium was replaced.

Fluorescence Microscopy

Pictures were taken using an IX71 fluorescence microscope (Olympus, Hamburg, Germany). Fluorescence dye Cerulean was detected at 433 nm emission/475 nm excitation.

Flow Cytometric Analysis

Flow cytometric data were acquired using the cytometer FACS-Calibur (488 nm laser; Becton Dickinson, Heidelberg, Germany). Staining of hMDR1-positive cells was carried out using an anti–P-glycoprotein antibody (Alexis Biochemicals through AXXORA Deutschland, Lörrach, Germany), followed by staining with a secondary PE-labeled antibody (R&D, Wiesbaden, Germany).

MTT Assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 96 hours of incubation modified as described before [6].

References

[1] Weber K, Bartsch U, Stocking C, and Fehse B (2008). A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol Ther* **16**, 698–706.

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[5] Dull T, Zufferey R, Kelly M, et al. (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**, 8463–8471.

[6] Michaelis M, Suhan T, Cinatl J, Driever PH, and Cinatl J, Jr (2004). Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. *Int J Oncol* **25**, 1795–1799.

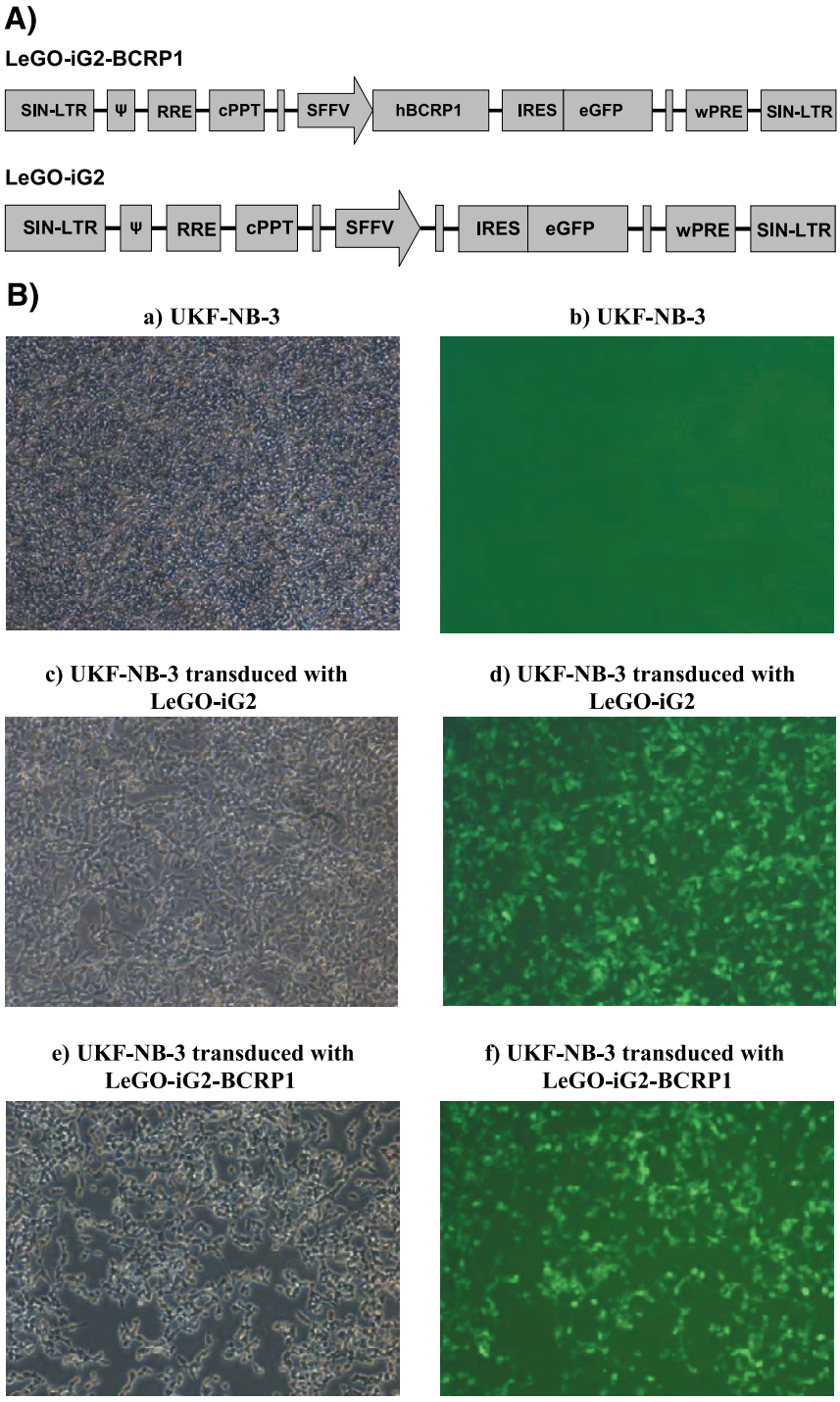
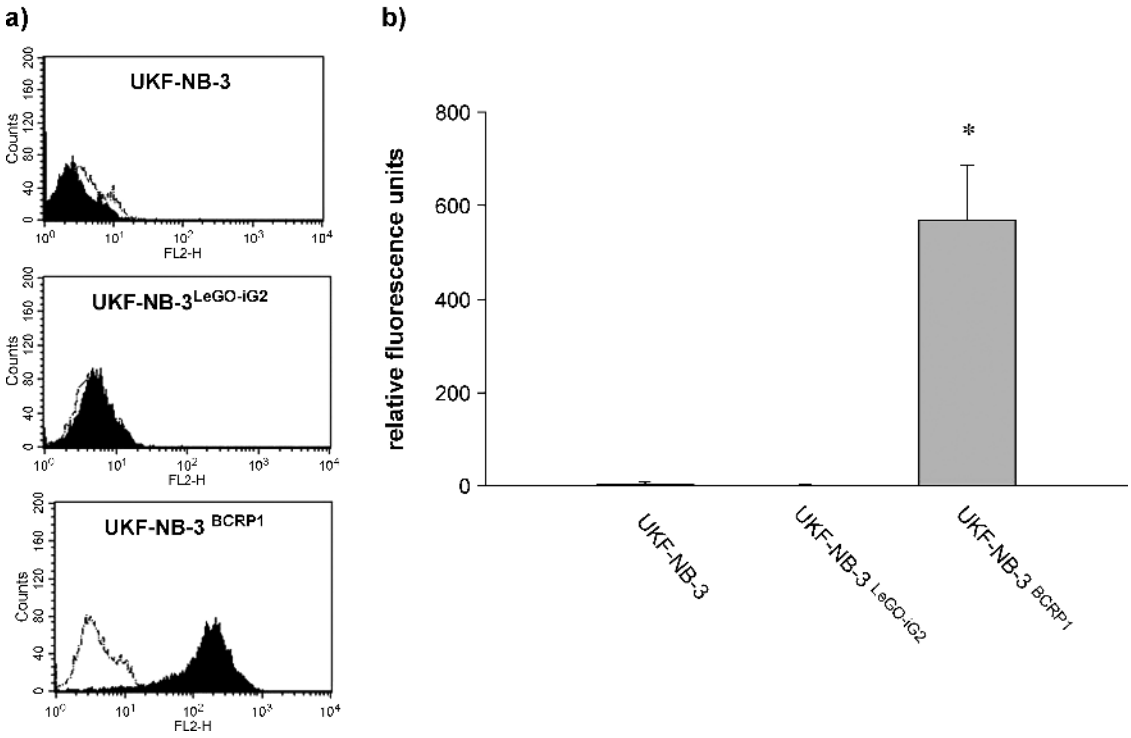
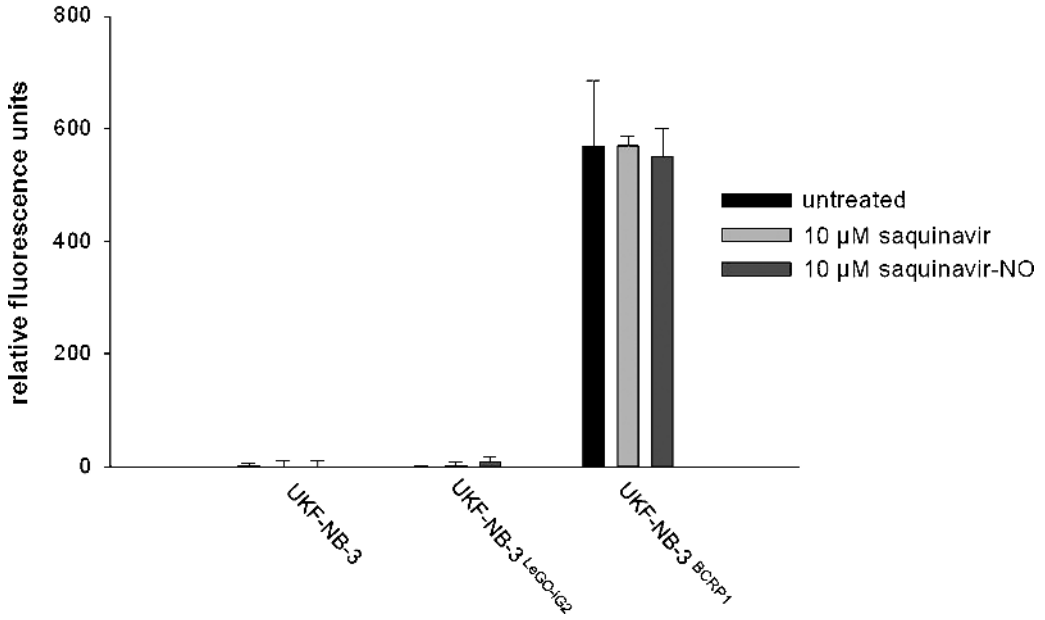


Figure W3. (A) Scheme of the lentiviral vectors, as integrated proviruses, that have been used in this study (not drawn to scale). Ψ indicates packaging signal; cPPT, central polypurine tract; eGFP, enhanced green fluorescent protein; hBCRP1, human breast cancer resistance protein 1 gene cDNA; IRES, internal ribosome entry site of the EMCV; RRE, rev-responsive element; SFFV, spleen focus–forming virus enhancer/promoter; SIN-LTR, self-inactivating long terminal repeat; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. (B) UKF-NB-3 cells, UKF-NB-3 cells transduced with LeGO-iG2 (UKF-NB-3$^{LeGO\text{-}iG2}$), and UKF-NB-3 cells transduced with LeGO-iG2-BCRP1 (UKF-NB-3^{BCRP1}) were photographed by inverse light microscopy (a, c, e) and by fluorescence microscopy (b, d, f) using an IX71 fluorescence microscope (Olympus). Fluorescence dye eGFP was detected at 484/507 nm (excitation/emission). The nontransduced control cell line UKF-NB-3 showed no fluorescence (b). In contrast, UKF-NB-3$^{LeGO\text{-}iG2}$ and UKF-NB-3^{BCRP1} cells displayed high fluorescence due to successful transduction with LeGO vectors (d, f). (C) Flow cytometric analysis of BCRP1 expression. (a) Flow cytometric histograms of the cell lines UKF-NB-3 (negative control), UKF-NB-3 transduced with 100 μl of control vector LeGO-iG2 (UKF-NB-3$^{LeGO\text{-}iG2}$) and UKF-NB-3 transduced with 100 μl of human BCRP1-expressing vector LeGO-iG2-BCRP1 (UKF-NB-3^{BCRP1}). (b) Quantitative analysis of flow cytometry data. *$P < .05$ relative to nontransduced UKF-NB-3 cells. (D) Flow cytometric analysis of BCRP1 expression in the cell lines UKF-NB-3, UKF-NB-3$^{LeGO\text{-}iG2}$, and UKF-NB-3^{BCRP1} untreated (black bars), treated with 10 μM saquinavir for 5 days (gray bars) or treated with 10 μM saq-NO for 5 days (dark gray bars). Incubation with saquinavir or saquinavir-NO had no influence on BCRP1 expression. (E) Concentrations that decrease cell viability by 50% (IC_{50}) for the BCRP1 substrate mitoxantrone, determined by MTT assay (values are means ± SD) in the absence or presence of the BCRP1 inhibitor WK-X-34. BCRP1-expressing cell line UKF-NB-3^{BCRP1} showed higher IC_{50}-values for mitoxantrone compared with BCRP1-negative cell lines UKF-NB-3 and UKF-NB-3$^{LeGO\text{-}iG2}$. [1]$P < .05$ relative to UKF-NB-3. [2]$P < .05$ relative to UKF-NB-3^{BCRP1}.

C)



D)



E)

Cell line	IC_{50} mitoxantrone (ng/ml)	IC_{50} mitoxantrone (ng/ml) + WK-X-34 (1µM)
UKF-NB-3	0.18 ± 0,084	0.16 ± 0.039
UKF-NB-3$^{LeGO-iG2}$	0.21 ± 0,056	0.23 ± 0.061
UKF-NB-3^{BCRP1}	45.87 ± 7,97[1]	0.17 ± 0.042[2]

Figure W3. (*continued*).

Materials and Methods of Viral Transduction

Cloning of Lentiviral Vectors

Standard molecular cloning techniques were used to generate viral vectors based on Lentiviral Gene Ontology (LeGO) vectors [1] (also http://www.LentiGO-Vectors.de). Maps and sequence data of the vectors are available on request. The lentiviral vector LeGO-iG2 was used as backbone vector. Then, the 1968-bp cDNA of the human *BCRP1* gene was cloned into LeGO-iG2 using *Bam*HI and *Not*I. The internal SFFV promoter of this vector transcribes a bicistronic mRNA with an IRES of the EMCV, expressing the hBCRP1 together with the enhanced green fluorescent protein (eGFP) as a marker. The vector LeGO-iG2 only expressing GFP served as a control.

Generation of Viral Particles

Cell-free viral supernatants were generated by transient transfection of 293T packaging cells as described [2], using the third-generation packaging plasmids pMDLg/pRRE and pRSV-Rev [3] together with phCMV-VSV-G [2]. Supernatants containing pseudotyped vector particles were titrated on 293T target cells. Gene transfer rates were analyzed 2 days after transduction by fluorescence-activated cell sorting (FACS). Titers of 2.4×10^6 (LeGO-iG2-BCRP1) and 5×10^6 (LeGO-iG2) VSV-G pseudotyped virus particles per milliliter of unconcentrated supernatants were obtained.

Cell Culture and Lentiviral Gene Transfer

All cells were cultured in their respective growth medium supplemented with penicillin/streptomycin. For transduction of UKF-NB-3 target cells, 5×10^4 cells in 500 µl of medium were plated per well in a 24-well plate. The next day, 250 µl of viral supernatant per well was added in the presence of 8 µg/ml polybrene, and the plate was centrifuged at 1000*g* for 1 hour at room temperature. After another 3 hours in the cell culture incubator, medium was replaced.

Fluorescence Microscopy

Pictures were taken using an IX71 fluorescence microscope (Olympus, Hamburg, Germany). Fluorescence dye eGFP was detected at 484 nm emission/507 nm excitation.

FACS Analysis

FACS data were acquired using the cytometer FACSCalibur (488 nm laser; Becton Dickinson, Heidelberg, Germany). Staining of hBCRP1-positive cells was carried out using an anti-hBCRP1 antibody (Kamiya Biomedical Company, Seattle, WA) followed by staining with a secondary PE-labeled antibody (R&D, Wiesbaden, Germany).

MTT Assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 96 hours of incubation modified as described before [4].

References

[1] Weber K, Bartsch U, Stocking C, and Fehse B (2008). A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol Ther* **16**, 698–706.

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