Reversal of P-glycoprotein–Mediated Multidrug Resistance by the Murine Double Minute 2 Antagonist Nutlin-3

Martin Michaelis,[1] Florian Rothweiler,[1] Denise Klassert,[1] Andreas von Deimling,[3] Kristoffer Weber,[2] Boris Fehse,[2] Bernd Kammerer,[4] Hans Wilhelm Doerr,[1] and Jindrich Cinatl, Jr.[1]

[1]Institut für Medizinische Virologie, Klinikum der J.W. Goethe-Universität and [2]Experimentelle Pädiatrische Onkologie und Hämatologie, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany; [3]Department of Neuropathology, Ruprecht-Karls-University Heidelberg and Deutsches Krebsforschungszentrum, Heidelberg, Germany; and [4]Institut für Pharmakologie und Toxikologie, Abteilung Klinische Pharmakologie, Klinikum der Eberhard-Karls-Universität, Tübingen, Germany

Abstract

Murine double minute 2 (MDM2) negatively regulates the activity of the tumor suppressor protein p53. Nutlin-3 is a MDM2 inhibitor under preclinical investigation as nongenotoxic activator of the p53 pathway for cancer therapy. Here, nutlin-3 was evaluated for its activity alone or in combination with established chemotherapeutic drugs for antitumor action in chemosensitive and chemoresistant neuroblastoma and rhabdomyosarcoma cell lines. Effects of nutlin-3 single treatment were much more pronounced in p53 wild-type cell lines (IC_{50}s <3 μmol/L) than in p53-mutated cell lines (IC_{50}s >17 μmol/L). In sharp contrast to the expectations, nutlin-3 concentrations that did not affect viability of p53-mutated cell lines strongly increased the efficacy of vincristine in p53-mutated, P-glycoprotein (P-gp)–overexpressing cell lines (decrease in IC_{50}s 92- to 3,434-fold). Similar results were obtained for other P-gp substrates. Moreover, nutlin-3 reduced efflux of rhodamine 123 and other fluorescence dyes that are effluxed by P-gp. Investigation of Madin-Darby canine kidney (MDCK) II cells stably transfected with plasmids encoding for P-gp (MDCKII MDR1) or multidrug resistance protein 1 (MRP-1, MDCKII MRP1) revealed that nutlin-3 not only interferes with P-gp but also affects MRP-1–mediated efflux. Kinetic studies and investigation of P-gp-ATPase activity showed that nutlin-3 is likely to act as a P-gp transport substrate. Examination of the nutlin-3 enantiomers nutlin-3a and nutlin-3b revealed that, in contrast to MDM2-inhibitory activity that is limited to nutlin-3a, both enantiomers similarly interfere with P-gp–mediated drug efflux. In conclusion, nutlin-3–induced inhibition of P-gp and MRP-1 was discovered as a novel anticancer mechanism of the substance in this report. [Cancer Res 2009;69(2):416–21]

Introduction

Murine double minute 2 (MDM2) negatively regulates the activity of the tumor suppressor protein p53. Nutlin-3 is a MDM2 inhibitor under preclinical investigation as nongenotoxic activator of the p53 pathway for cancer therapy (1) that also exerts antiangiogenic effects (2). Although the anticancer activity of nutlin-3 initially seemed to be limited to cells harboring wild-type p53 (1), recent results revealed that nutlin-3 also exerted anticancer effects in p53-negative and p53-mutant human tumor cells through E2F1 binding to MDM2 (3, 4).

In this report, nutlin-3 was investigated in combination with cytotoxic drugs in the pediatric cancer entities neuroblastoma and rhabdomyosarcoma. In p53 wild-type neuroblastoma cells, nutlin-3 caused apoptosis and/or neuronal differentiation (5) and increased sensitivity to chemotherapy-induced apoptosis (6). Information about the activity of nutlin-3 in rhabdomyosarcoma is lacking. Because nutlin-3 was shown to also exert anticancer effects that are independent of MDM2 inhibition (3), p53-mutated cells were included. Our most striking finding is that nutlin-3 sensitizes p53-mutated multidrug-resistant cancer cells to chemotherapy by interference with the ATP binding cassette (ABC) transporters P-glycoprotein [P-gp; encoded by multidrug resistance gene 1 (MDR1/ABCB1)] and multidrug resistance protein 1 (MRP-1/ABCC1).

Materials and Methods

Cell lines. The neuroblastoma cell lines UKF-NB-4 and UKF-NB-3 as well as the UKF-NB-3 sublines adapted to doxorubicin (20 ng/mL; UKF-NB-3^{r}DOX^{20}) or vincristine (10 ng/mL; UKF-NB-3^{r}VCR^{10}) were established as described before (7, 8). Be(2)-C cells were obtained from the American Type Culture Collection (ATCC). The alveolar rhabdomyosarcoma cell line UKF-Rhb-1 was established in our laboratory from a bone marrow metastasis. The alveolar rhabdomyosarcoma cell line Rh30 was kindly provided by Dr. P.J. Houghton (St. Jude's Children's Research Hospital, Memphis, TN). The vincristine-resistant rhabdomyosarcoma cell lines UKF-Rhb-1^{r}VCR^{10} and Rh30^{r}VCR^{10} were established by adaptation of UKF-Rhb-1 or Rh30 to growth in the presence of vincristine 10 ng/mL.

P53 status and P-gp expression status of the cell lines UKF-NB-3, UKF-NB-3^{r}DOX^{20}, UKF-NB-3^{r}VCR^{10}, UKF-NB-4, Be(2)-C, UKF-Rhb-1, UKF-Rhb-1^{r}VCR^{10}, Rh30, and Rh30^{r}VCR^{10} are shown in Table 1 (refs. 8–11; data for UKF-Rhb-1, UKF-Rhb-1^{r}VCR^{10} detected as described below).

The cell lines MDCKII MDR1 (12) and MDCKII MRP1 (12) and the nontransfected control cell line MDCKII were kind gifts of Drs. P. Borst, M. de Haas, and A.H. Schinkel (Nederlands Kanker Instituut, NKI, Amsterdam, the Netherlands). P53-negative, P-gp–negative PC-3 prostate cancer cells expressing low MRP-1 levels were received from ATCC. Adaptation of PC-3 to growth in the presence of vincristine 10 ng/mL resulted in a cell line that shows MRP-1 overexpression but no P-gp expression (PC-3^{r}VCR^{10}; data not shown).

All cell lines were propagated in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C.

Mutation analysis of p53. *TP53* gene sequencing on cDNAs was performed using the following four pairs of primers: TP53 Ex2-3-f

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Jindrich Cinatl, Institut für Medizinische Virologie, Paul Ehrlich-Str. 40, Frankfurt/Main 60596, Germany. Phone: 49-69-6301-6409; Fax: 49-69-6301-4302; E-mail: Cinatl@em.uni-frankfurt.de.

doi:10.1158/0008-5472.CAN-08-1856

Table 1. P-gp expression, p53-status, and tumor cell sensitivity to nutlin-3, vincristine, or nutlin-3 in combination with vincristine

Cell line	P-gp*	p53 mutation †	Nutlin-3 IC_{50} (μmol/L)	Vincristine IC_{50} (ng/mL)	Influence of nutlin-3 on the IC_{50} value for vincristine			
					Nutlin-3 (μmol/L)	Cell viability nutlin-3 (% control)	IC_{50} vincristine (ng/mL)	Fold reduction IC_{50} vincristine ‡
UKF-NB-3	−	−	1.16 ± 0.23	0.25 ± 0.03	0.25	92.05 ± 8.79	0.15 ± 0.02	1.67
UKF-NB-3^{r}VCR^{10}	+	+ (C135F)	> 20	72.25 ± 16.88	20	93.17 ± 11.23	0.17 ± 0.02	425
UKF-NB-3^{r}DOX^{20}	+	−	2.78 ± 0.51	198.52 ± 23.65	1	95.82 ± 13.51	6.87 ± 1.71	28.90
UKF-NB-4	+	+ (C175F)	19.69 ± 0.70	41.57 ± 8.32	5	100.14 ± 4.89	0.45 ± 0.10	92.38
Be(2)-C	+	+ (C135F)	> 20	37.77 ± 6.73	20	98.56 ± 6.42	0.011 ± 0.002	3434
UKF-Rhb-1	−	−	0.97 ± 0.11	0.37 ± 0.05	0.5	79.29 ± 12.61	0.23 ± 0.03	1.61
UKF-Rhb-1^{r}VCR^{10}	+	+ (K291X)	17.63 ± 2.24	42.67 ± 7.11	10	85.17 ± 9.88	0.26 ± 0.02	164
Rh30	−	+ (R273C)	> 20	0.30 ± 0.05	10	72.41 ± 11.69	0.09 ± 0.01	3.33
Rh30^{r}VCR^{10}	+	+ (R273C)	> 20	108.21 ± 16.42	10	71.04 ± 9.91	0.15 ± 0.03	721
Rh30^{MDR1}	+	+ (R273C)	> 20	15.43 ± 2.35	10	85.42 ± 9.28	0.14 ± 0.02	110
Rh30$^{empty\ vector}$	−	+ (R273C)	> 20	0.42 ± 0.15	10	82.36 ± 13.11	0.12 ± 0.03	3.50

*P-gp expression: +, overexpression; −, no overexpression.
† p53 status: +, mutated p53; −, wild-type p53.
‡ Ratio IC_{50} vincristine/IC_{50} vincristine in the presence of nutlin-3.

GTGACACGCTTCCCTGGAT and TP53 Ex2-3-r TCATCTGGACCTGGGTCTTC; TP53 Ex4-5-f CCCTTCCCAGAAAACCTACC and TP53 Ex4-5-r CTCCGTCATGTGCTGTGACT; TP53 EX6-7f GTGCAGCTGTGGGTTGATT and TP53 Ex6-7r GGTGGTACAGTCAGAGCCAAC; Tp53 Ex8-9-f CCTCACCATCATCACACTGG and TP53 Ex8-9-r GTCTGGTCCTGAAGGGTGAA. In addition, all cell lines were examined for TP53 mutations by sequence analysis of genomic DNA as described previously (13). PCR was performed as described before (8, 13). Each amplicon was sequenced bidirectionally.

Drugs. Vincristine was obtained from Sigma-Aldrich. Doxorubicin was from Cell Pharm. Nutlin-3, rhodamine 123, calcein-AM, and JC-1 were purchased from Merck Biosciences.

Viability assay. Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 96-h incubation modified as described before (14).

Real-time PCR. Total RNA was isolated from cell cultures using the TRI reagent (Sigma-Aldrich). Reverse transcription and real-time PCRs were performed as described before (15). The following primer sequences were used: 18s rRNA forward 5′-GTGAAACTGCGAATGGCTCAT, 18s rRNA reverse 5′-CTGACCGGGTTGGTTTTGAT, 18s rRNA probe 5′-(VIC) TGGTCGCTCCTCTCCCAC-(TAMRA), MDM2 forward 5′-TGTTGGTGCACAAAAAGACA, MDM2 reverse 5′-CACGCCAAACAAATCTCCTA, p21 forward 5′-GCCCGTGAGCGATGGAA, p21 reverse 5′-ACGCTCCCAGGCGAAGTC, GADD45 forward 5′-GCACGCCGCGCTCTCT, GADD45 reverse CTTATCCATCCTTTCGGTCTTCTG. The results are presented as fold change.

Flow cytometry and investigation of efflux of ABC transporter substrates. Antibodies directed against P-gp (Alexis Biochemicals via AXXORA Deutschland), MRP-1, MRP-3, or breast cancer resistance protein (BCRP/ABCG2, all three from Kamiya Biomedical Company) were used to detect protein expression by flow cytometry.

To investigate ABC transporter–mediated substance efflux, cells were incubated with rhodamine 123 (0.1 μmol/L, P-gp, MRPs), JC-1 (0.1 μmol/L, P-gp), or calcein-AM (2 μmol/L, MRPs) for 60 min. Then, cells were washed with PBS and incubated for another 60 min to allow ABC transporter–mediated drug efflux. Subsequently, cellular fluorescence was analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Rhodamine 123 was detected at the FL1 channel; JC-1 and calcein-AM were detected at the FL2 channel.

Determination of P-gp ATPase activity. The P-gp ATPase activity was determined using the Human PGP-ATPase Assay (BD Biosciences) following the manufacturer's instructions.

Viral transduction. Standard molecular cloning techniques were used to generate lentiviral vectors based on Lentiviral Gene Ontology (LeGO) vector technology[5] (ref. 16; Supplementary Fig. S1).

Separation of enantiomers. Nutlin-3 was separated in its enantiomers nutlin-3a and nutlin-3b by chiral reversed-phase chromatography (Supplementary Fig. S2).

Results

Influence of nutlin-3 on cancer cell viability. Nutlin-3 sensitivity markedly differed between p53 wild-type (<3 μmol/L) and p53 mutated cells (>17 μmol/L) after a 96-hour incubation (Table 1; Fig. 1*A*). Moreover, nutlin-3 (20 μmol/L) treatment induced expression of p53 target genes (*p21, MDM2, GADD45*) in p53 wild-type cells but not in p53-mutated cells as shown for UKF-NB-3, UKF-NB-3^{r}VCR^{10}, UKF-Rhb-1, and UKF-Rhb-1^{r}VCR^{10} cells by real-time PCR (Fig. 1*B* and *C*).

Efficacy of nutlin-3 in combination with anticancer drugs. Nutlin-3 was already shown to increase the cytotoxic activity of anticancer drugs (1). In UKF-NB-3 (p53-wild-type, P-gp-negative) cells, nutlin-3 and vincristine induced additive cytotoxicity (Fig. 2*A*). In sharp contrast to the results obtained with UKF-NB-3, nutlin-3 concentrations that were nontoxic to UKF-NB-3^{r}VCR^{10} cells (p53-mutated, high P-gp expression) dramatically increased the vincristine sensitivity of these cells (Fig. 2*A*). The combination of nutlin-3 and vincristine in concentrations that alone did not affect UKF-NB-3^{r}VCR^{10} cell viability caused complete destruction of all UKF-NB-3^{r}VCR^{10} cells (Fig. 2*A* and *C*). Similar results were obtained for the comparison of UKF-Rhb-1 cells (p53 wild-type,

[5] http://www.LentiGO-Vectors.de

P-gp-negative) and UKF-Rhb-1^{r}VCR^{10} (p53-mutated, high P-gp expression) cells (Fig. 2*B* and *C*). Investigation of further cell lines revealed that nontoxic or moderate toxic nutlin-3 concentrations only slightly decreased the IC_{50} concentration of vincristine in p53 wild-type and P-gp–negative cells (<2-fold; UKF-NB-3, UKF-Rhb-1), but massively decreased the IC_{50} of vincristine in p53-mutated and P-gp–positive cells [92- to 3,434-fold; UKF-NB-3^{r}VCR^{10}, UKF-Rhb-1^{r}VCR^{10}, UKF-NB-4; Be(2)-C; Table 1]. Moreover, nutlin-3 (10 μmol/L) induced a 3-fold reduction in the IC_{50} for vincristine in p53-mutated, P-gp negative Rh30 cells but caused a 721-fold reduction in the IC_{50} for vincristine in the P-gp–expressing vincristine-resistant subline Rh30^{r}VCR^{10} (Table 1). These findings suggest that nutlin-3 may interfere with P-gp–mediated vincristine efflux. In the p53 wild-type and P-gp–overexpressing cell line UKF-NB-3^{r}DOX^{20}, the IC_{50} for vincristine was decreased by 28.9-fold by nutlin-3 (1 μmol/L; Table 1). The reduced effect on vincristine sensitivity compared with other P-gp–overexpressing cell lines may be explained by the relatively low nutlin-3 concentration that had to be applied due to the higher nutlin-3 sensitivity of this p53 wild-type cell line.

Moreover, UKF-NB-3^{r}VCR^{10} or UKF-Rhb-1^{r}VCR^{10} cells were treated with nutlin-3 in combination with cytotoxic drugs with different modes of action that are structurally unrelated to vincristine. Nutlin-3 (20 μmol/L), a concentration that did not significantly affect UKF-NB-3^{r}VCR^{10} viability (96 ± 8% cell viability relative to untreated control), strongly sensitized UKF-NB-3^{r}VCR^{10} cells to the P-gp substrates doxorubicin, paclitaxel, etoposide, mitomycin C, and actinomycin D but not to cisplatin that is known not to be a P-gp substrate (Fig. 2*D*). Nutlin-3 (20 μmol/L) substantially decreased the IC_{50}s of doxorubicin (IC_{50}: 110 ± 13 ng/mL; IC_{50} + nutlin-3: 9 ± 1 ng/mL; fold decrease: 12.1), paclitaxel (IC_{50}: 51 ± 5 ng/mL; IC_{50} + nutlin-3: 1.5 ± 0.2 ng/mL; fold decrease: 33.6), etoposide (IC_{50}: 320 ± 47 ng/mL; IC_{50} + nutlin-3: 16 ± 2 ng/mL; fold decrease: 19.9), mitomycin C (IC_{50}: 34 ± 3 ng/mL; IC_{50} + nutlin-3: 4.6 ± 0.7 ng/mL; fold decrease: 7.5), or actinomycin D (IC_{50}: 22.3 ± 2.1 ng/mL; IC_{50} + nutlin-3: 0.34 ± 0.03 ng/mL; fold decrease: 65.7) in UKF-NB-3^{r}VCR^{10} cells but did not significantly affect sensitivity to cisplatin (IC_{50}: 178 ± 24 ng/mL; IC_{50} + nutlin-3: 145 ± 22 ng/mL; fold decrease: 1.2). Similar results were obtained in UKF-Rhb-1^{r}VCR^{10} cells (data not shown).

To investigate the effects of nutlin-3 in P-gp–expressing and non–P-gp-expressing cells that share the same genetic p53 background, p53-mutated, P-gp–negative Rh30 cells were transfected with lentiviral MDR1 vectors (see Supplementary Fig. S1). Transfection with empty vectors did not change the sensitivity of Rh30 (Rh30$^{empty\ vector}$) cells to nutlin-3, vincristine, or the combination of vincristine and nutlin-3 (Table 1). Rh30 cells transfected with MDR1 (Rh30^{MDR1}) showed decreased sensitivity to vincristine that was reverted by addition of nutlin-3.

Influence of nutlin-3 on the ABC transporter–mediated efflux of fluorescence substrates. The fluorescent P-gp substrate rhodamine 123 was used to investigate the influence of nutlin-3 on P-gp–mediated substance efflux. Rhodamine 123 (0.1 μmol/L) fluorescence was detected in P-gp–negative UKF-NB-3 cells but not in P-gp–overexpressing UKF-NB-3^{r}VCR^{10} cells (Fig. 3*A*). Nutlin-3 (20 μmol/L) treatment induced strong accumulation of rhodamine 123 in UKF-NB-3^{r}VCR^{10} cells (25-fold increase; Fig. 3*A*). In contrast, nutlin-3 (20 μmol/L) increased rhodamine 123 accumulation in UKF-NB-3 cells to a much lesser extent (1.7-fold increase; Fig. 3*A*). Noteworthy, nutlin-3–induced effects on rhodamine 123 accumulation were similar to those induced by verapamil that is known to inhibit P-gp being a P-gp substrate. Moreover, rhodamine 123 efflux inhibition by nutlin-3 occurred in a concentration-dependent

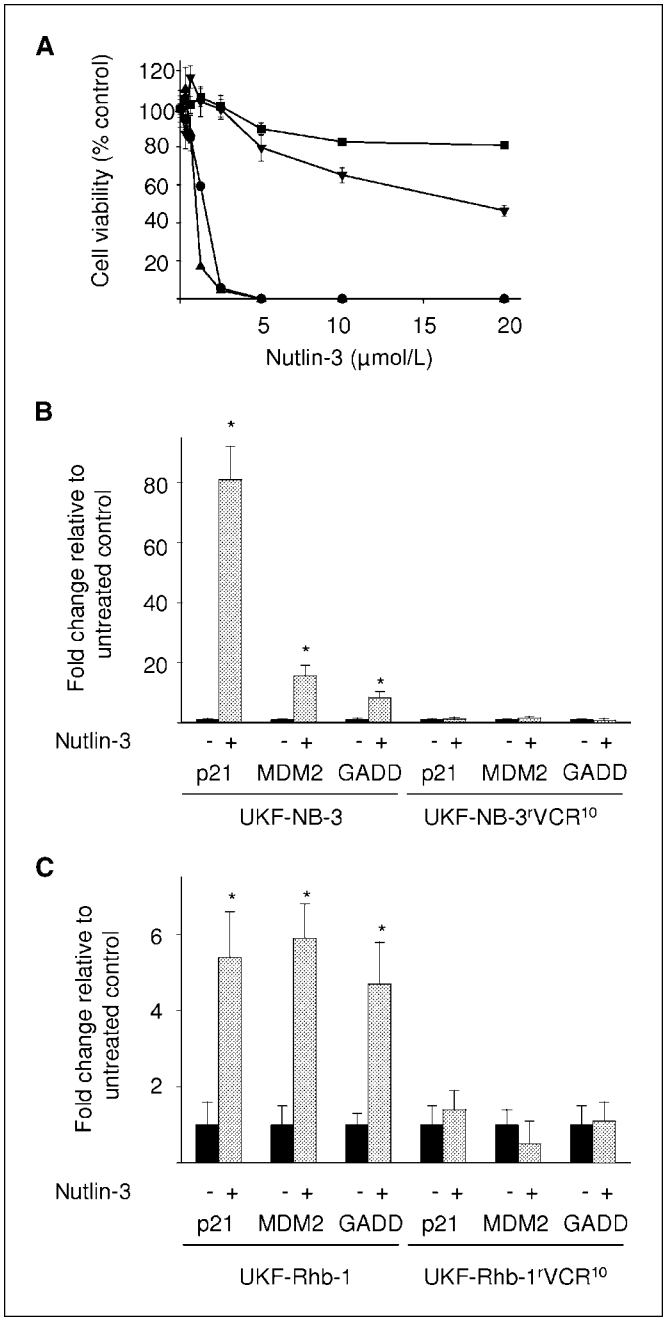

Figure 1. Influence of nutlin-3 on chemosensitive p53 wild-type (UKF-NB-3) or vincristine-resistant, p53-mutated, P-gp–overexpressing (UKF-NB-3^{r}VCR^{10}) neuroblastoma and chemosensitive, p53-wild type (UKF-Rhb-1) or vincristine-resistant, p53-mutated, P-gp–overexpressing (UKF-Rhb-1^{r}VCR^{10}) rhabdomyosarcoma cells. *A,* cell viability relative to nontreated control of nutlin-3–treated UKF-NB-3 (●), UKF-NB-3^{r}VCR^{10} (■), UKF-Rhb-1 (▲), or UKF-Rhb-1^{r}VCR^{10} (▼) cells determined by the MTT assay after 96-h incubation; *B,* expression of the p53 target genes *p21, MDM2*, and *GADD45* (*GADD*) in nutlin-3 (20 μmol/L)–treated UKF-NB-3 or UKF-NB-3^{r}VCR^{10} cells after 8 h detected by real-time PCR. *C,* expression of the p53 target genes *p21, MDM2*, and *GADD45* (*GADD*) in nutlin-3 (20 μmol/L)–treated UKF-Rhb-1 or UKF-Rhb-1^{r}VCR^{10} cells after 8 h detected by real-time PCR. *, $P < 0.05$ compared with untreated control.

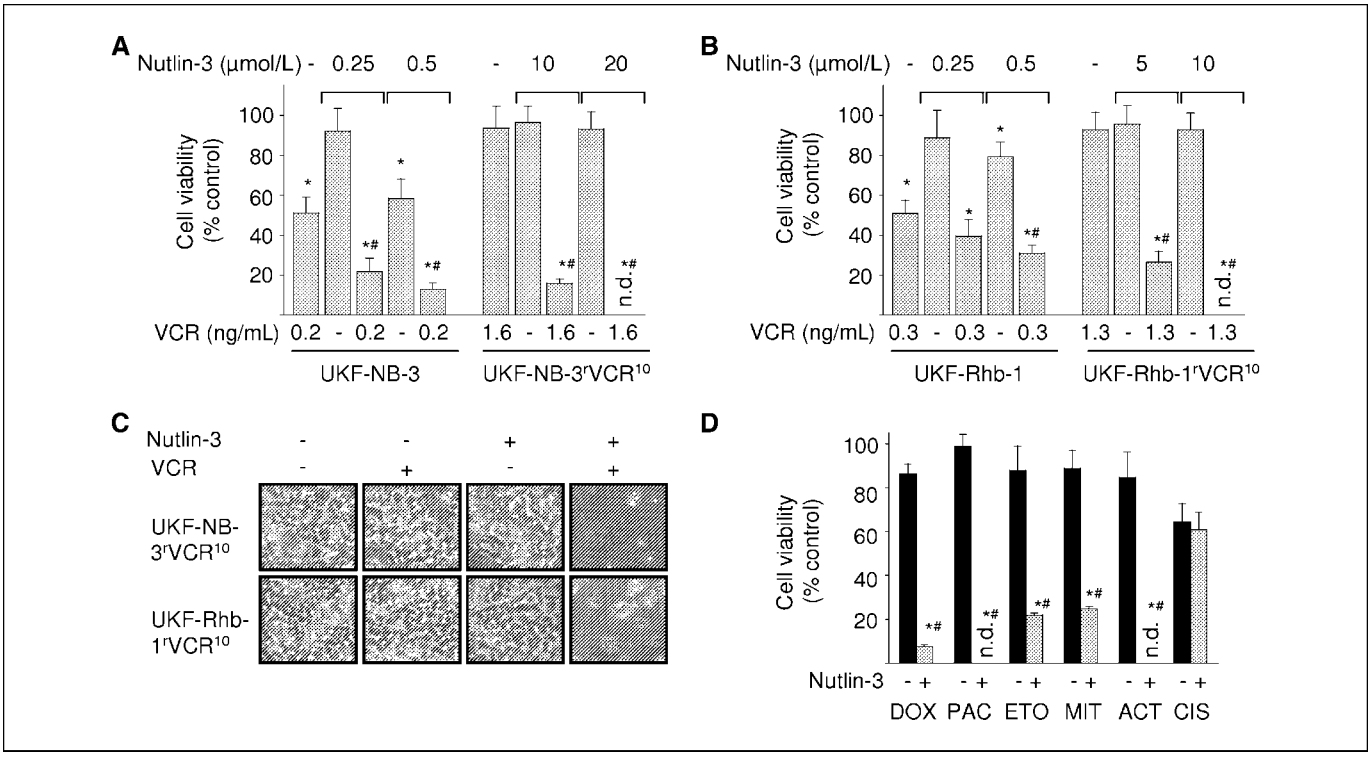

Figure 2. Influence of nutlin-3 on vincristine cytotoxicity in cancer cells. *A,* viability of chemosensitive, p53 wild-type (UKF-NB-3) or vincristine-resistant, p53-mutated, P-gp–overexpressing (UKF-NB-3^{r}VCR^{10}) neuroblastoma cells treated with vincristine (*VCR*), nutlin-3, or combinations of vincristine and nutlin-3. *B,* viability of chemosensitive, p53 wild-type (UKF-Rhb-1), or vincristine-resistant, p53-mutated, P-gp–overexpressing (UKF-Rhb-1^{r}VCR^{10}) rhabdomyosarcoma cells treated with vincristine, nutlin-3, or combinations of vincristine and nutlin-3. *C,* representative photographs showing UKF-NB-3^{r}VCR^{10} cells treated for 48 h without or with vincristine (5 ng/mL), nutlin-3 (20 μmol/L), or vincristine (5 ng/mL) plus nutlin-3 (20 μmol/L) or UKF-Rhb-1^{r}VCR^{10} cells treated for 48 h without or with vincristine (5 ng/mL), nutlin-3 (10 μmol/L), or vincristine (5 ng/mL) plus nutlin-3 (10 μmol/L). *D,* influence of nutlin-3 (20 μmol/L) in a concentration that does not significantly affect UKF-NB-3^{r}VCR^{10} cell viability (96 ± 8% viability relative to control) on antitumor effects of the P-gp substrates doxorubicin (*DOX*) 50 ng/mL, paclitaxel (*PAC*) 12.5 ng/mL, etoposide (*ETO*) 100 ng/mL, mitomycin C (*MIT*) 12.5 ng/mL, actinomycin D (*ACT*) 12.5 ng/mL, or the non–P-gp substrate cisplatin (*CIS*) 100 ng/mL. *, $P < 0.05$ compared with untreated control; #, $P < 0.05$ compared with either single treatment; *n.d.,* no viable cells detected.

manner in UKF-NB-3^{r}VCR^{10} cells (Fig. 3*B*). Investigation of the P-gp substrate JC-1 that is structurally not related to rhodamine 123 resulted in similar results: accumulation in UKF-NB-3 cells but not in UKF-NB-3^{r}VCR^{10} cells and strong inhibition of JC-1 efflux by nutlin-3 in UKF-NB-3^{r}VCR^{10} cells (data not shown).

MRP-1 is another family member of the ABC transporters that was shown to be involved in cancer cell chemoresistance (17, 18). P-gp plays a dominant role between the ABC transporters in UKF-NB-3^{r}VCR^{10} cells. No significant overexpression of MRP-3 or BCRP could be detected. Moreover, MRP-specific (MK571) or BCRP-specific (fumitremorgin C) inhibitors did not significantly affect efflux of different fluorescence substrates from UKF-NB-3^{r}VCR^{10} cells (data not shown). Accumulation of rhodamine 123 that is a substrate of P-gp and MRP-1 was measured in MDCKII cells stably overexpressing P-gp (MDCKII MDR1) or MRP-1 (MDCKII MRP1) with or without nutlin-3 treatment. Results indicated decreased rhodamine 123 accumulation in MDCKII MDR1 and in MDCKII MRP-1 cells. Moreover, nutlin-3 caused strong accumulation of rhodamine 123 in both cell lines (Fig. 3*C*). The P-gp inhibitor verapamil (5 μg/mL) showed comparable effects like nutlin-3 in MDCKII MDR1 cells and the specific MRP inhibitor MK571 (20 μmol/L) showed comparable effects like nutlin-3 in MDCKII MRP1 cells (data not shown). Nutlin-3 inhibited rhodamine 123 efflux from MDCKII MDR1 or MDCKII MRP1 cells in a concentration-dependent manner, showing still weak effects in a concentration of 1.25 μmol/L but no effect in a concentration of 0.625 μmol/L anymore (data not shown). Similar results were obtained by the use of the fluorescence dye calcein-AM in MDCKII MRP1 cells (data not shown). To investigate the influence of nutlin-3 on MRP-1 in a second model, nutlin-3 was examined in PC-3^{r}VCR^{10}, the vincristine-resistant subline of the p53-negative PC-3 cell line, showing high MRP-1 expression but no P-gp expression. In PC-3^{r}VCR^{10} cells, nutlin-3 (20 μmol/L) decreased the IC_{50} value for vincristine from 45.78 ± 3.87 ng/mL to 15.45 ± 2.21 ng/mL. The MRP inhibitor MK571 (20 μmol/L) reduced it to 11.57 ± 0.45 ng/mL. Moreover, nutlin-3 caused calcein-AM accumulation similarly to the MRP inhibitor MK571 (20 μmol/L) in PC-3^{r}VCR^{10} cells (Supplementary Fig. S3).

Nutlin-3 is transported by P-gp. The question if nutlin-3 may act as direct inhibitor of P-gp function or as P-gp substrate was addressed by two different approaches. First, the time kinetics of nutlin-3–mediated inhibition of rhodamine 123 efflux was investigated in UKF-NB-3^{r}VCR^{10} and MDCKII MDR1 cells. Whereas P-gp inhibitors commonly show prolonged activity even after a washing-out period, the activity of P-gp substrates rapidly declines after substance removal (19, 20). To examine this, cells were incubated with rhodamine 123 in the presence of nutlin-3 (20 μmol/L) or verapamil (5 μg/mL), which is a P-gp substrate, for 60 minutes. Then, cells were washed and rhodamine 123 fluorescence was detected after 0, 15, 30, 60, and 120 minutes. Results indicated that rhodamine 123 fluorescence rapidly decreases after wash-out of nutlin-3 or verapamil (Fig. 3*D*).

To further investigate if a substance may inhibit P-gp or act as P-gp substrate, the P-gp ATPase activity can be determined (19, 20). The P-gp ATPase activity is often stimulated in the presence of transported substrates such as verapamil. Determination of the P-gp ATPase activity revealed that nutlin-3 (20 μmol/L) and verapamil (5 μg/mL) stimulated P-gp ATPase activity to a similar extent and no antagonism between verapamil and nutlin-3 in stimulation of P-gp ATPase activity (Fig. 3*D*). Taken together, these results suggest that nutlin-3 acts as a P-gp transport substrate.

Influence of the nutlin-3 enantiomers nutlin-3a and nutlin-3b on P-gp. To investigate if the nutlin-3 enantiomers nutlin-3a and nutlin-3b exert differential effects on P-gp, their influence was tested on UKF-NB-3^{r}VCR^{10} cell viability in combination with vincristine (Supplementary Table S1), on rhodamine 123 efflux in UKF-NB-3^{r}VCR^{10} cells, and on P-gp ATPase activity (Supplementary Fig. S4). Results revealed that both enantiomers showed similar effects on P-gp function.

Discussion

In this report, the MDM2 inhibitor nutlin-3 was investigated alone or in combination with chemotherapeutic drugs in a panel of chemosensitive and chemoresistant neuroblastoma and rhabdomyosarcoma cell lines. In concordance with previous studies (5), nutlin-3 was much more effective in p53 wild-type than in p53-mutated neuroblastoma cells. Moreover, we could show that the anticancer activity of nutlin-3 also includes p53 wild-type rhabdomyosarcoma cells. A previous report had shown that nutlin-3 sensitizes p53 wild-type neuroblastoma cells to chemotherapy-induced cell death (6). We found that nutlin-3 interferes with P-gp function being a P-gp substrate. Nontoxic nutlin-3 concentrations strongly decreased the IC_{50} for vincristine (and other P-gp substrates structurally nonrelated to vincristine) in different p53-mutated, P-gp–expressing cell lines (92- to 3,434-fold).

In addition to P-gp, other ABC transporters may play a role in cancer cell chemoresistance (17, 18). Our results indicate that nutlin-3 also interferes with MRP-1 activity. Systematic analysis of

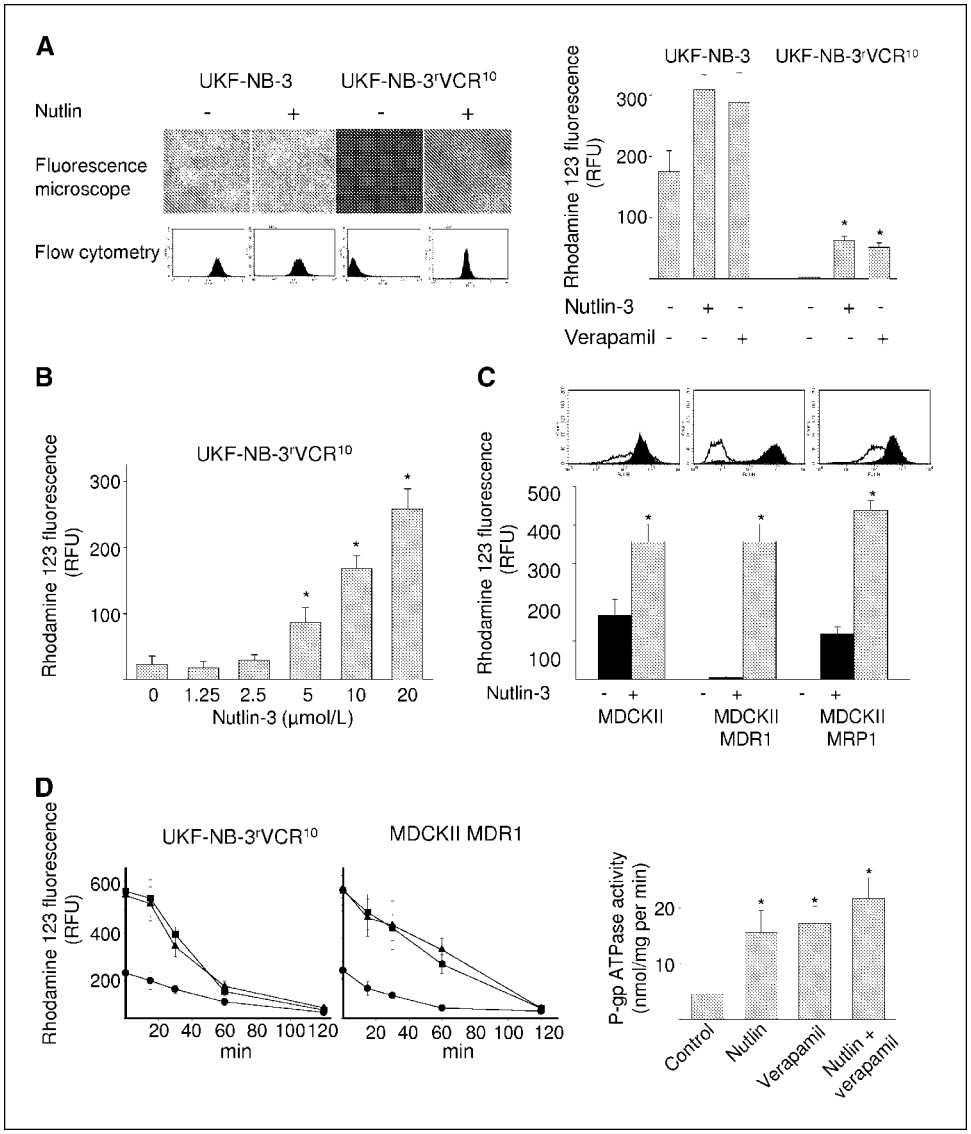

Figure 3. Influence of nutlin-3 on cellular accumulation of ABC transporter substrates. *A,* representative photographs and representative flow cytometry experiments showing rhodamine 123 (0.1 μmol/L) fluorescence in p53 wild-type (UKF-NB-3) or vincristine-resistant, p53-mutated, P-gp–overexpressing (UKF-NB-3^{r}VCR^{10}) neuroblastoma cells; influence of nutlin-3 (20 μmol/L) or the P-gp transport substrate verapamil (5 μg/mL) on rhodamine 123 fluorescence in UKF-NB-3 or UKF-NB-3^{r}VCR^{10} neuroblastoma cells determined by flow cytometry. *B,* concentration-dependent influence of nutlin-3 on rhodamine 123 fluorescence in UKF-NB-3^{r}VCR^{10} neuroblastoma cells. *C,* representative flow cytometry experiments showing rhodamine 123 (2 μmol/L) accumulation in MDCKII cells, MDCKII cells stably transfected with MDR-1 encoding for P-gp (MDCKII MDR1), or MDCKII cells stably transfected with MRP-1 (MDCKII MRP1) without (*white peaks*) or with (*black peaks*) nutlin-3 (20 μmol/L) treatment; rhodamine (2 μmol/L) accumulation in MDCKII, MDCKII MDR1, or MDCKII MRP1 cells without or with nutlin-3 (20 μmol/L) treatment determined by flow cytometry. *D,* time kinetics of rhodamine 123 fluorescence in UKF-NB-3^{r}VCR^{10} cells (0.1 μmol/L rhodamine 123) or MDCKII MDR1 cells (2 μmol/L rhodamine 123) after incubation for 60 min with rhodamine 123 (●), nutlin-3 (20 μmol/L), and rhodamine 123 (■), or verapamil (5 μg/mL) and rhodamine 123 (▲) and wash-out. P-gp ATPase activity in isolated membranes in the presence of nutlin-3 (20 μmol/L), verapamil (5 μg/mL), or their combination (*Control,* activity in untreated membranes). *, $P < 0.05$ compared with untreated control.

the influence of nutlin-3 on the activity of different ABC transporters is warranted. Notably, nutlin-3 is a racemate with nutlin-3a being the enantiomer that inhibits MDM2, whereas nutlin-3b does not (1). Both enantiomers similarly interfere with P-gp, indicating that the underlying structure-activity relationships differ from those defined for MDM2 inhibition.

In conclusion, our data show that nutlin-3 interferes with P-gp– or MRP-1–mediated drug efflux in multidrug-resistant cancer cells, representing a novel anticancer mechanism of this substance. This newly discovered activity requires further consideration during the development of this drug. Moreover, previous findings about synergisms between nutlin-3 and cytotoxic drugs may be reanalyzed concerning the role of ABC transporters. Because overexpression of ABC transporters and namely P-gp belongs to the most important chemoresistance mechanisms, nutlin-3 may be a new lead structure for the design of ABC transporter inhibitors.

Disclosure of Potential Conflicts of Interest

J. Cinatl, H.W. Doerr, and M. Michaelis have filed a patent application for the use of nutlin-3 as an ABC-transporter inhibitor. The other authors declared no potential conflicts of interest.

Acknowledgments

Received 5/20/2008; revised 9/18/2008; accepted 10/14/2008.

Grant support: Hilfe für krebskranke Kinder Frankfurt e.V. and their foundation Frankfurter Stiftung für krebskranke Kinder (M. Michaelis, F. Rothweiler, D. Klassert, H.W. Doerr, and J. Cinatl). The work was in part supported by the EU-funded project SYNLET (contract No. 043312).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the friendly society "Hilfe für krebskranke Kinder Frankfurt e.V." and its foundation "Frankfurter Stiftung für krebskranke Kinder" for support; Eva Bechtold, Janette Spitznagel, Lena Stegmann, and Jochen Meyer for their skillful technical assistance; Dr. U. Delabar, Institute of Pharmacology and Toxicology, Department of Clinical Pharmacology (University Hospital Tübingen), for her analytic advice and her assistance in sample collection of nutlin-3a and nutlin-3b; and Axel Schambach and Christopher Baum, MH Hannover, Germany, for providing the MDR1 cDNA.

References

1. Vassilev LT. MDM2 inhibitors for cancer therapy. Trends Mol Med 2007;13:23–31.
2. Secchiero P, Corallini F, Gonelli A, et al. Antiangiogenic activity of the MDM2 antagonist nutlin-3. Circ Res 2007; 100:61–9.
3. Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, Schwartz GK. Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. Oncogene 2007;26:3473–81.
4. Secchiero P, Zerbinati C, Melloni E, et al. The MDM-2 antagonist nutlin-3 promotes the maturation of acute myeloid leukemic blasts. Neoplasia 2007;9:853–61.
5. Van Maerken T, Speleman F, Vermeulen J, et al. Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma. Cancer Res 2006;66:9646–55.
6. Barbieri E, Mehta P, Chen Z, et al. MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. Mol Cancer Ther 2006;5:2358–65.
7. Cinatl J, Jr., Cinatl J, Kotchetkov R, et al. Bovine seminal ribonuclease selectively kills human multidrug-resistant neuroblastoma cells via induction of apoptosis. Int J Oncol 1999;15:1001–9.
8. Kotchetkov R, Driever PH, Cinatl J, et al. Increased malignant behavior in neuroblastoma cells with acquired multi-drug resistance does not depend on P-gp expression. Int J Oncol 2005;27:1029–37.
9. Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J. Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. Cancer Res 2001;61:8–13.
10. Michaelis M, Cinatl J, Anand P, et al. Onconase induces caspase-independent cell death in chemoresistant neuroblastoma cells. Cancer Lett 2007;250:107–16.
11. Hosoi H, Dilling MB, Shikata T, et al. Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells. Cancer Res 1999;59:886–94.
12. Bakos E, Evers R, Szakács G, et al. Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. J Biol Chem 1998;273: 32167–75.
13. Hayashi Y, Ueki K, Waha A, Wiestler OD, Louis DN, von Deimling A. Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme. Brain Pathol 1997;7:871–5.
14. Michaelis M, Suhan T, Cinatl J, Driever PH, Cinatl J, Jr. Valproic acid and interferon-α synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. Int J Oncol 2004;25:1795–9.
15. Cinatl J, Jr., Michaelis M, Fleckenstein C, et al. West Nile virus infection induces interferon signalling in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2006;47:645–51.
16. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. Mol Ther 2008;16:698–706.
17. Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.
18. Calcagno AM, Kim IW, Wu CP, Shukla S, Ambudkar SV. ABC drug transporters as molecular targets for the prevention of multidrug resistance and drug-drug interactions. Curr Drug Deliv 2007;4:324–33.
19. Ludwig JA, Szakács G, Martin SE, et al. Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. Cancer Res 2006;66:4808–15.
20. Newman MJ, Rodarte JC, Benbatoul KD, et al. Discovery and characterization of OC144–093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance. Cancer Res 2000;60:2964–72.