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Adaptation of cancer cells from different entities to the MDM2 inhibitor nutlin-3 results in the emergence of p53-mutated multi-drug-resistant cancer cells

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Six p53 wild-type cancer cell lines from infrequently p53-mutated entities (neuroblastoma, rhabdomyosarcoma, and melanoma) were continuously exposed to increasing concentrations of the murine double minute 2 inhibitor nutlin-3, resulting in the emergence of nutlin-3-resistant, p53-mutated sublines displaying a multi-drug resistance phenotype. Only 2 out of 28 sublines adapted to various cytotoxic drugs harboured p53 mutations. Nutlin-3-adapted UKF-NB-3 cells (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, harbouring a G245C mutation) were also radiation resistant. Analysis of UKF-NB-3 and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells by RNA interference experiments and lentiviral transduction of wild-type p53 into p53-mutated UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells revealed that the loss of p53 function contributes to the multi-drug resistance of UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells. Bioinformatics PANTHER pathway analysis based on microarray measurements of mRNA abundance indicated a substantial overlap in the signalling pathways differentially regulated between UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ and UKF-NB-3 and between UKF-NB-3 and its cisplatin-, doxorubicin-, or vincristine-resistant sublines. Repeated nutlin-3 adaptation of neuroblastoma cells resulted in sublines harbouring various p53 mutations with high frequency. A p53 wild-type single cell-derived UKF-NB-3 clone was adapted to nutlin-3 in independent experiments. Eight out of ten resulting sublines were p53-mutated harbouring six different p53 mutations. This indicates that nutlin-3 induces *de novo* p53 mutations not initially present in the original cell population. Therefore, nutlin-3-treated cancer patients should be carefully monitored for the emergence of p53-mutated, multi-drug-resistant cells.

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The tumour suppressor protein p53 is the so-called guardian of the genome that is activated in response to (genotoxic) stress, resulting in cell cycle inhibition and DNA repair or cell death. Many anti-cancer drugs are suspected to induce their anti-cancer effects, at least in part, through induction of p53. The important role of p53 as tumour suppressor is illustrated by the fact that *TP53*, the gene encoding for p53, is the most frequently mutated gene in cancer cells. About 50% of all tumours are characterised by cancer cells in which p53 function is damaged through expression of mutated proteins or loss of expression. Moreover, in many cell lines harbouring wild-type p53, p53 response is impaired due to changes in its up- and/or downstream signalling pathways.[1]

Currently, numerous cancer treatment approaches are dedicated to the activation of p53 in p53 wild-type cancer cells.[1] One approach is to interfere with the interaction of p53 and its regulator murine double minute 2 (MDM2, in humans also called HDM2). When p53 is activated, it transcriptionally induces MDM2 expression via binding to the P2 promoter of the *MDM2* gene. MDM2 physically interacts with p53, thereby inhibiting its transactivation function, mediating p53 export from the nucleus, and inducing proteasomal degradation of p53.[1,2]

Nutlin-3 is a prototype, small-molecule MDM2 inhibitor and non-genotoxic activator of p53.[1,2] Initially, it was hoped that the so-called non-genotoxic p53 activators, such as nutlin-3, that do not target the DNA integrity and exert less nonspecific toxicity than the traditional cytotoxic drugs may cause less genomic instability and result in reduced formation of resistant cells. This hope was challenged by some recent findings. Nutlin-3 was shown to induce DNA damage probably by MDM2 inhibition-dependent and -independent mechanisms.[3,4]

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Abbreviations: CDDP, cisplatin; DAC, dacarbazine; DOCE, docetaxel; DOX, doxorubicin; FDR, false discovery rate; GEMCI, gemcitabine; IRINO, irinotecan; MDM2, murine double minute 2; MEL, melphalan; OXALI, oxaliplatin; PCL, paclitaxel; RCCL collection, resistant cancer cell line collection; TOPO, topotecan; VCR, vincristine; VINOR, vinorelbine

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Moreover, nutlin-3 treatment of U2OS osteosarcoma cells and HCT116 colon cancer cells resulted in the emergence of tetraploid, nutlin-3-resistant cells. In nutlin-3-treated SJSA-1 osteosarcoma cells, p53-mutated cells emerged.[5,6]

Here, we investigated the nutlin-3-induced resistance formation in a panel of neuroblastoma, rhabdomyosarcoma, and melanoma cells. Nutlin-3 had already been shown to exert anti-cancer effects in neuroblastoma, rhabdomyosarcoma, and melanoma cells.[7–12] All of these entities are characterised by relatively low frequencies of p53 mutation,[8,13–16] making them possible candidates for nutlin-3 treatment. Our results show that nutlin-3-adaptation results with high frequency in the acquisition of p53 mutations in originally p53 wild-type cells. In general, p53-mutated nutlin-3-resistant cells display a multi-drug-resistant phenotype. Transcriptomics and subsequent bioinformatics pathway analysis suggested an overlap in the resistance-associated pathways in cells adapted to nutlin-3 and those adapted to cytotoxic anti-cancer drugs. Results from the adaptation of a single wild-type p53 cell-derived clone of the neuroblastoma cell line UKF-NB-3 indicate that nutlin-3 induces *de novo* p53 mutations.

Results

Continuous exposure of p53 wild-type neuroblastoma, rhabdomyosarcoma, and melanoma cell lines to nutlin-3 results in the establishment of p53-mutated, multi-drug-resistant sublines. Continuous exposure to increasing nutlin-3 concentrations for 6–13 passages (Supplementary Table 1) of the neuroblastoma cell lines UKF-NB-3, UKF-NB-2, and UKF-NB-6, the rhabdomyosarcoma cell line UKF-Rhb-1, and the melanoma cell lines Colo-679 and Mel-HO resulted in the formation of p53-mutated sublines (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$: G245C (homozygote); UKF-NB-2^{r}Nutlin$^{10\,\mu M}$: G117W (heterozygote); UKF-NB-6^{r}Nutlin$^{10\,\mu M}$: K132N (heterozygote), P223L (homozygote); UKF-Rhb-1^{r}Nutlin$^{10\,\mu M}$: D281N (homozygote); Colo-679^{r}Nutlin$^{20\,\mu M}$: C238F (heterozygote); Mel-HO^{r}Nutlin$^{20\,\mu M}$: R248Q (heterozygote)). The p53 mutations became detectable in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells after five passages in the presence of nutlin-3, in UKF-Rhb-1^{r}Nutlin^{10} cells after four passages, in Colo-679^{r}Nutlin$^{20\,\mu M}$ cells after four passages, and in Mel-HO^{r}Nutlin$^{20\,\mu M}$ cells after three passages.

In general, the nutlin-3-adapted cell lines showed decreased sensitivity to various anti-cancer drugs (Table 1; Supplementary Table 2).

Repeated adaptation of UKF-NB-3 and UKF-NB-6 cell lines to nutlin-3. To investigate whether the adaptation of cancer cells to nutlin-3 and the accompanying emergence of p53 mutations are reproducible, 9 additional nutlin-3-resistant UKF-NB-3 sublines and 10 additional nutlin-3-resistant UKF-NB-6 cell sublines were established (Table 2). Adaptation times ranged from 8 to 14 passages, indicating repeatability of the process in both the cell lines (Supplementary Table 1). Out of the nine nutlin-3-resistant UKF-NB-3 sublines, four harboured p53 mutations (three times D281G, one time P278A, Table 2). All 10 additional nutlin-3-resistant UKF-NB-6 sublines harboured various p53 mutations (Table 2).

Repeated adaptation of a single p53 wild-type cell-derived UKF-NB-3 clone to nutlin-3 results in sublines harbouring varying p53 mutations. The emergence of p53-mutated cells during nutlin-3 adaptation may be the consequence of the selection of a preexisting p53-mutated sub-population or nutlin-3 may favour the formation of novel p53 mutations in p53 wild-type cells. A total of 10 single cell clones of UKF-NB-3 were established and all were found to be p53 wild-type. Next, one single p53 wild-type cell-derived UKF-NB-3 clone was adapted to growth in the presence of nutlin-3 in 10 independent experiments. A total of 7–11 passages were needed for nutlin-3 adaptation (Supplementary Table 3). Eight of the resulting ten sublines harboured six different p53 mutations (Table 3).

P53 status in cytotoxic drug-adapted cell lines. A number of sublines of the investigated cell lines adapted to different cytotoxic drugs were analysed for their p53 status. Among UKF-NB-3 sublines adapted to 11 different cytotoxic drugs (UKF-NB-3^{r}CDDP^{1000} cisplatin (CDDP);

Table 1 Sensitivity of UKF-NB-3 and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells to a panel of established cytotoxic drugs indicated by the concentration that reduces cell viability by 50% (IC_{50}[a]) indicated by MTT assay after a 5-day treatment period

	IC_{50} (ng/ml[a])			
		UKF-NB-3^{r}Nutlin$^{10\,\mu M}$		
Drug	**UKF-NB-3**	**After 7 passages of adaptation to nutlin-3**	**After 20 passages of adaptation to nutlin-3**	**After 7 passages of adaptation to nutlin-3 plus 20 passages cultivation in the absence of nutlin-3**
Nutlin-3[a]	1.01 ± 0.11	35.09 ± 4.21	38.71 ± 5.60	34.88 ± 6.75
Vincristine	0.29 ± 0.04	1.41 ± 0.33	1.32 ± 0.26	1.36 ± 0.20
Cisplatin	108.77 ± 22.32	293.02 ± 31.63	342.52 ± 42.71	280.14 ± 24.83
Actinomycin D	0.20 ± 0.05	0.59 ± 0.18	0.70 ± 0.24	0.61 ± 0.18
Melphalan	178.69 ± 52.47	439.56 ± 46.71	451.02 ± 51.31	426.22 ± 44.89
Paclitaxel	1.33 ± 0.31	3.48 ± 0.42	3.25 ± 0.18	3.09 ± 0.41
Doxorubicin	9.18 ± 1.64	24.21 ± 4.03	28.41 ± 3.97	29.97 ± 5.86
Topotecan	0.90 ± 0.06	3.95 ± 1.09	4.22 ± 0.85	3.77 ± 0.68

[a]All concentrations are expressed in ng/ml, except nutlin-3 concentrations that are expressed in μM

Table 2 p53 status of UKF-NB-3 and UKF-NB-6 sublines adapted to nutlin-3 in independent experiments

UKF-NB-3 sublines	p53 status	UKF-NB-6 sublines	p53 status
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$	G245C[a,b]	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$	K132N[c], P223L[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ I	Wild type	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ I	S241F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ II	Wild type	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ II	C277F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ III	Wild type	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ III	C277F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ IV	Wild type	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ IV	C135F[c], D281Y[c]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ V	Wild type	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ V	C277F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ VI	P278A[c]	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ VI	R248Q[c]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ VII	D281G[c]	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ VII	C277F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ VIII	D281G[c]	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ VIII	C277F[b]
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ IX	D281G[c]	UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ IX	C277F[b]
		UKF-NB-6^{r}Nutlin$^{10\,\mu M}$ X	R248Q[b]

[a]Type of p53 mutation
[b]Homozygote
[c]Heterozygote

Table 3 p53 status in nutlin-3-adapted sublines of the p53 wild-type UKF-NB-3 clone 2

Subline	p53 status
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$I	Wild type
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$II	Wild type
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$III	H168R (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$IV	H168R (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$V	C277F (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$VI	H168R (heterozygote), G245S (homozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$VII	F270L (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$VIII	H168R (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$IX	R280S (heterozygote), D281G (heterozygote)
UKF-NB-3clone2^{r}Nutlin$^{10\,\mu M}$X	N239D (heterozygote)

UKF-NB-3^{r}DAC^{8} dacarbazine (DAC); UKF-NB-3^{r}DOX^{20} doxorubicin (DOX); UKF-NB-3^{r}GEMCI^{10} gemcitabine (GEMCI); UKF-NB-3^{r}IRINO^{800} irinotecan (IRINO); UKF-NB-3^{r}MEL^{1000} melphalan (MEL); UKF-NB-3^{r}OXALI^{2000} oxaliplatin (OXALI); UKF-NB-3^{r}PCL^{20} paclitaxel (PCL); UKF-NB-3^{r}TOPO^{15} topotecan (TOPO); UKF-NB-3^{r}VCR^{10} vincristine (VCR); and UKF-NB-3^{r}VINOR^{20} vinorelbine (VINOR); Supplementary Table 4), only UKF-NB-3^{r}VCR^{10} showed a p53 mutation (C135F, heterozygote) that was different from the G245C mutation observed in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells. Among the eight UKF-Rhb-1 sublines adapted to anti-cancer drugs (UKF-Rhb-1^{r}CDDP^{1000} CDDP; UKF-Rhb-1^{r}DOCE^{10} docetaxel (DOCE); UKF-Rhb-1^{r}DOX^{10} DOX; UKF-Rhb-1^{r}GEMCI^{10} GEMCI; UKF-Rhb-1^{r}IRINO^{200} IRINO; UKF-Rhb-1^{r}MEL^{400} MEL; UKF-Rhb-1^{r}OXALI^{1000} OXALI; and UKF-Rhb-1^{r}VCR^{10} VCR; Supplementary Table 4), only the UKF-Rhb-1^{r}VCR^{10} subline harboured a p53 mutation (K291X, heterozygote) that differed from the D281N mutation detected in UKF-Rhb-1^{r}Nutlin$^{10\,\mu M}$ cells. No p53 mutations were found in the UKF-NB-2 (UKF-NB-2^{r}CDDP^{1000}, UKF-NB-2^{r}DOX^{20}, and UKF-NB-2^{r}VCR^{10}) or melanoma sublines (Colo-679^{r}VCR^{20}, Colo-679^{r}PLX4032$^{10\,\mu M}$, MelHO^{r}VCR^{10}, MelHO^{r}CDDP^{1000}, MelHO^{r}DAC^{20}, and MelHO^{r}PLX4032$^{10\,\mu M}$; Supplementary Table 4). These data do not suggest the acquisition of p53 mutations to be a common event in these cell lines.

Sensitivity of UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells to cytotoxic drugs and radiation. In order to further characterise the resistance phenotype induced by adaptation to nutlin-3, the UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were investigated in more detail. In addition to their decreased sensitivity to a broad panel of different anti-cancer drugs (Table 1), UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells showed a strongly reduced sensitivity to irradiation (Figure 1). Among the investigated cell lines, the chemoresistant UKF-NB-3 sublines (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, UKF-NB-3^{r}VCR^{10}, UKF-NB-3^{r}DOX^{20}, and UKF-NB-3^{r}CDDP^{1000}) generally showed a decreased sensitivity to irradiation compared with the parental UKF-NB-3 cells.

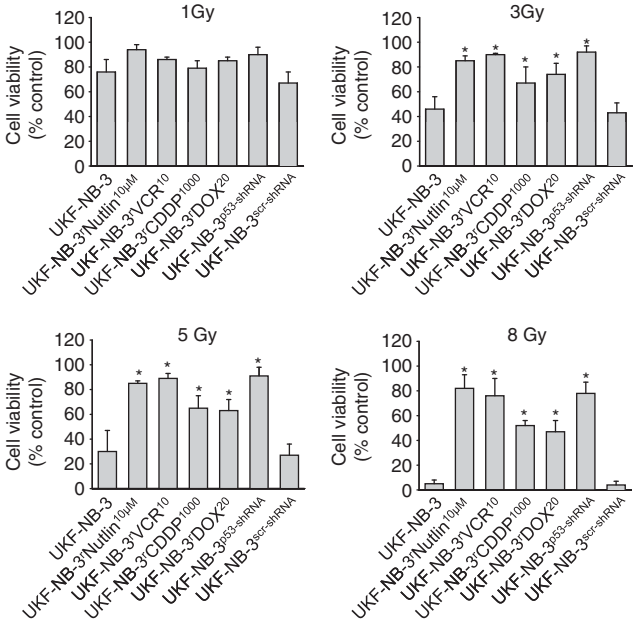

Figure 1 Radiation sensitivity of the UKF-NB-3 cell line, its sublines resistant to nutlin-3 (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$), VCR (UKF-NB-3^{r}VCR^{10}), CDDP (UKF-NB-3^{r}CDDP^{1000}), or DOX (UKF-NB-3^{r}DOX^{20}), and UKF-NB-3 cells transfected with lentiviral vectors expressing shRNA directed against p53 (UKF-NB-3$^{p53-shRNA}$) or scrambled shRNA (UKF-NB-3$^{scr-shRNA}$). A total of 1×10^{4} cells were seeded per well in 96-well plates, allowed to adhere for about 5 h, irradiated, and viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h post irradiation. *$P < 0.05$ relative to UKF-NB-3

Investigation of caspase 3/7 activation in response to cytotoxic drugs revealed that apoptosis induction is reduced and delayed in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells compared with UKF-NB-3 (Figure 2).

Previous investigations had shown that transient treatment of osteosarcoma cells and colorectal cancer cells with nutlin-3 may result in the formation of tetraploid clones. Tetraploid clones of HCT116 showed decreased sensitivity to CDDP and γ-irradiation.[5] However, in nutlin-3-treated UKF-NB-3 neuroblastoma cells, no formation of tetraploid cells was detected, whereas nutlin-3 treatment of SJSA sarcoma cells resulted in detectable tetraploidy (Supplementary Figure 1). In contrast, treatment of UKF-NB-3 cells with the aurora kinase inhibitor VX680 (tozasertib), a positive control known to induce tetraploidy, resulted in the formation of tetraploid cells.

Role of p53 mutation in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cell chemo- and radiation resistance. In concordance with its action on MDM2, nutlin-3 was shown to be specifically cytotoxic to p53 wild-type neuroblastoma and rhabdomyosarcoma cells.[7,11] P53 mutations may not only be associated with a loss of function but also with a gain of oncogenic functions, increasing tumour aggressiveness and metastatic potential, as well as drug resistance.[17] Therefore, the role of the G245C mutation in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells was further elucidated in the context of their sensitivity/resistance to anticancer drugs. Nutlin-3 or CDDP induced expression of the p53 target genes *CDKN1A* (encoding for p21), *PMAIP1* (encoding for NOXA), *MDM2*, and *GADD45* in p53 wild-type UKF-NB-3 cells but not in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells (Supplementary Figure 2A), indicating that p53 lost its function as transcription factor in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells. Accordingly, chromatin immunoprecipitation experiments revealed a lack of p53 binding to the promoters of p21, PMAIP1, or GADD45 promoters in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells (Supplementary Figure 2B).

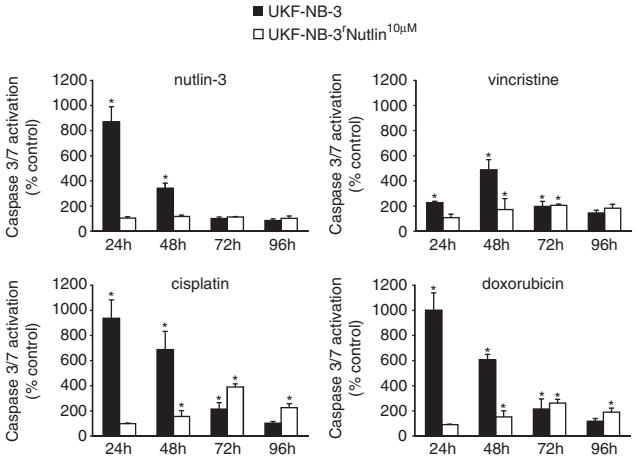

Figure 2 Caspase 3/7 activation in UKF-NB-3 or UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells treated nutlin-3 (10 μM), VCR (10 ng/ml), DOX (20 ng/ml), or CDDP (1000 ng/ml). $^{*}P<0.05$ relative to non-treated control

Next, p53 expression was suppressed in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells using a lentiviral vector expressing p53 shRNA, resulting in the subline UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{p53-shRNA}}$ (see Rothweiler *et al*;[18] Supplementary Figure 3). The chemosensitivity profile of UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{p53-shRNA}}$ cells did not differ from that of UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells (Supplementary Table 5), suggesting that the G245C mutation does not result in a gain of function of p53 in the context of cancer cell chemoresistance in this cell system.

UKF-NB-3 cells transduced with a lentiviral vector expressing p53 shRNA (UKF-NB-3$^{\text{p53-shRNA}}$, Rothweiler *et al*[18]) were used to investigate the influence of loss of p53-function on chemosensitivity of UKF-NB-3 cells. UKF-NB-3$^{\text{p53-shRNA}}$ cells showed a decreased sensitivity to a range of different anticancer drugs (Supplementary Table 2) in comparison with UKF-NB-3 cells and UKF-NB-3$^{\text{scr-shRNA}}$ cells (control transduced with a vector expressing scrambled shRNA). This indicates that loss of p53 function is associated with decreased sensitivity of UKF-NB-3 cells to cytotoxic therapy.

Moreover, p53 appears to have a dominant role in the response of UKF-NB-3 cells to irradiation. Although all investigated chemoresistant UKF-NB-3 cell lines were less sensitive to irradiation than the parental UKF-NB-3 cells, resistance was more pronounced in the p53-mutated cell lines (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ and UKF-NB-3^{r}VCR^{10}) than in the p53 wild-type cells (UKF-NB-3^{r}DOX^{20} and UKF-NB-3^{r}CDDP^{1000}). In addition, UKF-NB-3$^{\text{p53-shRNA}}$ cells were similar irradiation resistant like the p53-mutated cell lines UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ and UKF-NB-3^{r}VCR^{10} (Figure 1).

Finally, UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were transduced with a lentiviral vector encoding for wild-type p53 (UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{wtp53}}$ Supplementary Figure 4). UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{wtp53}}$ cells showed increased sensitivity to nutlin-3 and cytotoxic drugs (Table 4), as well as to radiation (Figure 3) compared with UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells or UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells transduced with a control vector. However, their sensitivity to nutlin-3 was still reduced when compared with that of the parental UKF-NB-3 cells.

UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells remain resistant against multiple drugs after long-time cultivation in the absence of nutlin-3. Previous reports had suggested that treatment of different cancer cell lines with cytotoxic drugs may lead to

Table 4 Sensitivity of UKF-NB-3 cells, UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells, and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells transduced with a lentiviral vector encoding wild-type p53 (UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{wtp53}}$), UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells transduced with a control vector (UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{control}}$) to nutlin-3 or established cytotoxic drugs indicated by the concentration that reduces cell viability by 50% (IC_{50}^{1}) indicated by MTT assay after a 5-day treatment period

Cell line	Nutlin-3[a]	Vincristine	Paclitaxel	Topotecan
UKF-NB-3	0.95 ± 0.16	0.20 ± 0.02	0.92 ± 0.26	0.90 ± 0.08
UKF-NB-3^{r}Nutlin$^{10\,\mu M}$	39.12 ± 2.82	1.29 ± 0.13	2.98 ± 0.70	3.30 ± 0.14
UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{wtp53}}$	3.31 ± 0.28	0.48 ± 0.04	1.62 ± 0.11	1.23 ± 0.14
UKF-NB-3^{r}Nutlin$^{10\,\mu M\text{control}}$	41.52 ± 10.18	1.34 ± 0.18	2.95 ± 0.63	3.47 ± 0.46

[a]All concentrations are expressed in ng/ml, except nutlin-3 concentrations that are expressed in μM

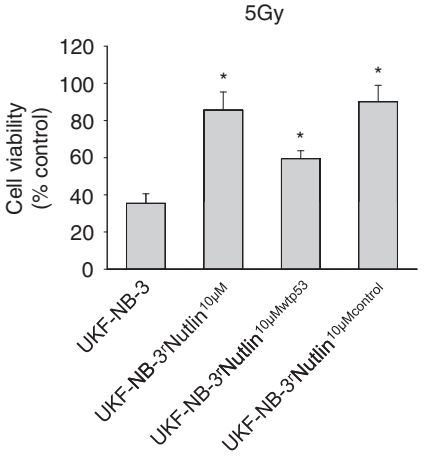

Figure 3 Radiation sensitivity of the UKF-NB-3 cell line, its sublines resistant to nutlin-3 (UKF-NB-3^{r}Nutlin$^{10\,\mu M}$), UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells transduced with a lentiviral vector expressing wild-type p53 (UKF-NB-3^{r}Nutlin$^{10\,\mu M wtp53}$), or UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells transduced with control vector (UKF-NB-3^{r}Nutlin$^{10\,\mu M control}$). A total of 1×10^4 cells were seeded per well in 96-well plates, allowed to adhere for about 5 h, irradiated (5 Gy), and viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h post irradiation. *$P < 0.05$ relative to UKF-NB-3

a selection of drug-resistant cancer cells.[20,21] After withdrawal of cytotoxic drugs, these cell lines returned to their chemosensitive phenotype. However, UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells retained their chemoresistance phenotype after 20 passages in the absence of nutlin-3 (Table 1), indicating that adaptation to nutlin-3 does not cause the reversible enrichment of a p53-mutated cancer cell fraction.

Transcriptomics analysis of gene expression in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells. In order to get an idea of the number of changes that result from the adaptation of UKF-NB-3 cells to nutlin-3 at the transcriptome level, we compared gene expression at the mRNA level between the parental UKF-NB-3 cell line and its sublines UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, UKF-NB-3^{r}VCR^{10} UKF-NB-3^{r}CDDP^{1000}, and UKF-NB-3^{r}DOX^{20} by gene microarray analysis (Supplementary Table 6). The number of significantly differentially expressed genes (false discovery rate (FDR) <0.05, corrected for multiple testing) varied between 3657 (UKF-NB-3 *versus* UKF-NB-3^{r}Nutlin$^{10\,\mu M}$) and 6375 (UKF-NB-3 *versus* UKF-NB-3^{r}CDDP^{1000}) being less than a twofold difference. The expression of 465 genes was significantly differentially regulated in the same direction (i.e., up or down) between UKF-NB-3 and each drug-adapted subline (Supplementary Table 6). Comparison of the number of genes that were significantly differentially regulated in the same direction in three resistant sublines relative to UKF-NB-3 revealed the highest overlap in the three cell lines adapted to the established cytotoxic drugs VCR, CDDP, and DOX (1571 genes), whereas the overlaps between UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells and the other cell lines resulted in lower consistencies (596–790 genes; Supplementary Table 6). Similar results were obtained for the overlaps of genes significantly differentially regulated in the same direction in two resistant sublines compared with UKF-NB-3 (Supplementary Table 6).

Bioinformatics pathway analysis using the PANTHER database revealed that expression of genes from 8 (UKF-NB-3 *versus* UKF-NB-3^{r}Nutlin$^{10\,\mu M}$) to 18 (UKF-NB-3 *versus* UKF-NB-3^{r}VCR^{10}) signal transduction pathways significantly differed ($P < 0.05$, corrected for multiple testing) between the UKF-NB-3 cell line and the drug-adapted sublines (Supplementary Table 7). The three most significantly affected signalling pathways between UKF-NB-3 and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were 'p53 pathway feedback loops 2', 'Wnt signalling', and 'p53 pathway'. Notably, the overall overlap was very high, with seven of the eight pathways significantly differentially regulated between UKF-NB-3 and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were also found to be significantly differentially regulated between UKF-NB-3 cells and UKF-NB-3^{r}CDDP^{1000}, UKF-NB-3^{r}VCR^{10}, or UKF-NB-3^{r}DOX^{20} cells (Supplementary Table 7).

Discussion

Nutlin-3 inhibits the physical interaction between p53 and its inhibitor MDM2, and activates p53 responses in p53 wild-type cancer cells.[1,2] Neuroblastomas, rhabdomyosarcomas, and melanomas are known to be rarely p53 mutated,[8,13–16] and therefore considered as promising candidates for p53-activating agents. In this report, continuous exposure of p53 wild-type cells from a panel of neuroblastoma, rhabdomyosarcoma, and melanoma cell lines to increasing nutlin-3 concentrations led to the emergence of nutlin-3-resistant sublines within total 28 out of 35 nutlin-3-adapted sublines harbouring p53 mutations. Investigation of 28 cytotoxic drug-adapted sublines of the investigated cell lines revealed only two p53-mutated cell lines (UKF-NB-3^{r}VCR^{10} and UKF-Rhb-1^{r}VCR^{10}). This indicates that induction of p53 mutation is a rare event during chemoresistance acquisition in these cell lines.

All of the p53 mutations detected in nutlin-3-adapted cells are missense mutations in the DNA binding domain of p53. Thus, they represent the same type of p53 aberration that is most commonly found in tumour samples and cancer cell lines. Indeed, all but one (G117W) of the mutations of p53 that we report here to be generated upon nutlin-3 adaptation have been previously found in patient biopsies and established cancer cell lines (see http://www-p53.iarc.fr/), confirming that the continuous treatment with nutlin-3 confers a strong selection pressure against functional wild-type p53. Interestingly, however, although we find mutations affecting the so-called hotspot codons (i.e., example G245 and R248) – these are among the p53 residues most frequently found mutated in tumours – we have also detected 'rare' mutations that have been reported in only a very small number of tumour samples and cell lines (i.e., P223L, S241F, and R280S). The properties of such mutated p53 proteins are not well characterised. However, given that the residues affected lye within the DNA-binding domain of p53, it seems likely that these mutations also impair the transactivation function of the tumour suppressor protein.

The nutlin-3-adapted p53-mutated cell lines UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, UKF-NB-2^{r}Nutlin$^{10\,\mu M}$, UKF-NB-6^{r}Nutlin$^{10\,\mu M}$, UKF-Rhb-1^{r}Nutlin$^{10\,\mu M}$, Colo-679^{r}Nutlin$^{20\,\mu M}$, and Mel-HO^{r}Nutlin$^{20\,\mu M}$ displayed multi-drug resistance phenotypes, that is,

they were less sensitive to a panel of different anti-cancer drugs than their parental counterparts. Particular in neuroblastomas, loss of p53 function has been correlated to chemoresistance, more aggressive disease, and poor prognosis.[14,16,21,22]

UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, the nutlin-3-adapted subline of the neuroblastoma cell line UKF-NB-3, was investigated more in detail. UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells harboured a G245C mutation. UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells showed also a profound resistance to radiation. An analysis of UKF-NB-3, UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, and a UKF-NB-3 subline in which p53 was depleted due to transduction with a lentiviral vector encoding p53 shRNA indicated that the loss of p53 function contributes to the multi-drug-resistant phenotype observed in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells. In addition, bioinformatics PANTHER pathway analysis based on transcriptomics data derived from mRNA detection using gene microarray revealed a substantial overlap in the signalling pathways significantly differentially regulated ($P < 0.05$ after correction for multiple testing) between UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ and UKF-NB-3 cells, as well as between those significantly differentially regulated between UKF-NB-3 and UKF-NB-3^{r}CDDP^{1000}, UKF-NB-3^{r}DOX^{20} and UKF-NB-3^{r}VCR^{10} cells. CDDP concentrations that were cytotoxic to UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells did neither induce expression of *p53* target genes nor binding of p53 to the promoters of selected target genes. Moreover, apoptosis induction as indicated by caspase activation was reduced and delayed in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells relative to UKF-NB-3 cells. As inhibition of p53 expression in UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells did not affect their sensitivity to the investigated anti-cancer drugs, the G245C mutation appears to be a loss-of-function mutation and not to be associated with the gain of oncogenic functions in the context of chemoresistance in this cell system.

The ability of mutant p53 forms to inactivate wild-type p53 may differ in dependence of the type of mutation and also in dependence of the specific conditions.[23] G245C-mutated p53 harbouring UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were sensitised to toxicity induced by nutlin-3, cytotoxic drugs, and radiation by transduction with a lentiviral vector expressing wild-type p53 (UKF-NB-3^{r}Nutlin$^{10\,\mu Mwtp53}$) However, UKF-NB-3^{r}Nutlin$^{10\,\mu Mwtp53}$ cells remained less sensitive to nutlin-3, cytotoxic drugs, or radiation than parental UKF-NB-3 cells. It remains unclear to which extent these differences are caused by a wild-type p53-inactivating function of G245C-mutated p53 and/or by other changes acquired during the adaptation of UKF-NB-3 cells to nutlin-3.

The emergence of p53-mutated, drug-resistant cells under nutlin-3 exposure raises the question where these cells originate from. Cytotoxic drugs had already been shown to exert a selection pressure on certain cancer cell lines, resulting in the enrichment of a of drug-resistant cancer cell fraction.[19,20] After removal of the cytotoxic drugs, these cell lines returned to their original chemosensitive phenotype. As UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells retained their chemoresistant phenotype even after 20 passages of cultivation in the absence of nutlin-3, nutlin-3 does not appear to reversibly enhance a certain cellular fraction present in the parental UKF-NB-3 cell line.

Nevertheless, nutlin-3 treatment may still result in the (irreversible) expansion of p53-mutated cell fractions already present in the parental UKF-NB-3 cell line. In a previous report, nutlin-3 treatment of SJSA-1 osteosarcoma cells was shown to result in the emergence of p53-mutated cells.[6] The authors used a different approach (pulsed nutlin-3 exposure) and detected different p53 mutations in different experiments.[6] These findings are in concordance with our results from the repeated adaptation of UKF-NB-3 and UKF-NB-6 neuroblastoma cells to nutlin-3 that also showed that various p53 mutations emerge. On the basis of the varying p53 mutations in nutlin-3-selected SJSA-1 cells and the fact that they did not receive growing cell colonies under continuous nutlin-3 exposure, they suggested that the p53 mutations are likely to arise during nutlin-3 treatment.[6] However, this interpretation might not be conclusive, as nutlin-3 was shown to also inhibit the growth of cells without functional p53 by mechanisms involving p73 and E2F1.[24,25] Moreover, cells were adapted by continuous nutlin-3 exposure in our experiments. Differences in the used cell lines may be responsible for this discrepancy. The same group had also previously shown that nutlin-3 treatment of U2OS osteosarcoma and HCT116 colon carcinoma cells may promote endoreduplication and emergence of nutlin-3-resistant tetraploid cells.[5] In our experiments, we detected tetraploid SJSA-1 cells but did not find tetraploid UKF-NB-3 cells. This supports the assumption that different cells and/or cell types may clearly react differently to nutlin-3 treatment.

The emergence of various p53 mutations after repeated adaptation of a cell line to nutlin-3 may also not be sufficient to conclude that nutlin-3 induces p53 mutations, as cancer cell lines and tumours may harbour various p53 mutations.[26–28] In order to investigate whether nutlin-3 induces p53 mutations or selects p53-mutated cell populations that are already present in the original cell line, a p53 wild-type single cell-derived clone of UKF-NB-3 was established and adapted to nutlin-3 in 10 independent experiments. Eight out of ten nutlin-3-adapted sublines harboured six different p53 mutations, indicating nutlin-3 to induce the emergence of novel p53 mutations that were not present in the original cell population.

The mechanisms by which nutlin-3 favours p53 mutations remain unclear. Nutlin-3 was shown to induce and/or promote DNA damage.[3,4] Moreover, we show that the numbers of genes significantly differentially expressed ($P<0.05$ after correction for multiple testing) between UKF-NB-3 and UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ (3657) do not too strongly differ from those differentially expressed between UKF-NB-3 and the cytotoxic drug-adapted cell lines UKF-NB-3^{r}CDDP^{1000} (CDDP adapted, 6375), UKF-NB-3^{r}DOX^{20} (DOX adapted, 4711), and UKF-NB-3^{r}VCR^{10} (VCR adapted, 5885), especially considering the fact that UKF-NB-3^{r}Nutlin$^{10\,\mu M}$ cells were only cultivated for a few passages in parallel with the parental UKF-NB-3 cells, whereas the establishment of the other cell lines took more than a year. Therefore, nutlin-3 treatment does not appear to induce much lesser changes to cancer cells than less specific cytotoxic drugs. Moreover, more specific targeted therapies appear to be in general associated with a relatively rapid resistance development.[29,30] Consequently, interference with DNA integrity and a specific selection pressure favouring p53 loss-of-function mutations may be involved in the emergence of p53-mutated cells under nutlin-3 exposure.

In conclusion, our results show that nutlin-3 treatment of a panel of cell lines from three different entities (neuroblastoma,

rhabdomyosarcoma, and melanoma) known to be rarely p53-mutated and therefore considered as promising candidates for nutlin-3 therapy reproducibly resulted in the irreversible emergence of p53-mutated, multi-drug-resistant sublines. In contrast to previous reports, nutlin-3 did not induce formation of tetraploid cells. Induction of p53 mutation appears to be an exclusive property of nutlin-3, as only 2 out of 28 neuroblastoma, rhabdomyosarcoma, and melanoma cell lines adapted to various cytotoxic anti-cancer drugs developed p53 mutations. Repeated adaptation of a single p53-wild type cell-derived UKF-NB-3 clone in 10 independent experiments resulted in eight sublines harbouring six different p53 mutations and two p53 non-mutated sublines, indicating that nutlin-3 induces *de novo* p53 mutations and does not select preexisting p53-mutated sub-populations already existent in the original cell line. Although continuous nutlin-3 treatment may not completely reflect the tumour exposure to nutlin-3 in a clinical setting, cancer patients treated with nutlin-3 (and possibly also other MDM2 inhibitors or non-genotoxic p53 activators) should be carefully monitored for the emergence of p53-mutated, multi-drug-resistant cells.

Materials and Methods

Drugs. Nutlin-3 was purchased from Selleck Chemicals via BIOZOL GmbH (Eching, Germany). VCR, CDDP, and PCL were obtained from TEVA GmbH (Radebeul, Germany). DOX was received from Medac Gesellschaft für klinische Spezialpräparate mbH (Hamburg, Germany). MEL and TOPO were purchased from GlaxoSmithKline GmbH and Co. KG (Munich, Germany). Actinomycin D was received from MSD Sharp and Dome GmbH (Haar, Germany).

Cell lines. The N-myc-amplified neuroblastoma cell lines UKF-NB-2, UKF-NB-3, and UKF-NB-6 were established from stage 4 neuroblastoma patients.[31–33] The alveolar rhabdomyosarcoma cell line UKF-Rhb-1 was established from a bone marrow metastasis.[11] The melanoma cell lines Colo-679 and Mel-HO were obtained from the DSMZ (Braunschweig, Germany).

Parental chemosensitive cell lines were adapted to growth in the presence of anti-cancer drugs by continuous exposure of these cell lines to the increasing concentrations of these drugs as described before.[31,32]

The following chemoresistant UKF-NB-3 sublines were derived from the resistant cancer cell line (RCCL) collection: UKF-NB-3^{r}CDDP^{1000} (adapted to CDDP), UKF-NB-3^{r}DAC^{8} (DAC), UKF-NB-3^{r}DOX^{20} (DOX), UKF-NB-3^{r}GEMCI^{10} (GEMCI), UKF-NB-3^{r}IRINO^{800} (IRINO), UKF-NB-3^{r}MEL^{400} (MEL), UKF-NB-3^{r}OXALI^{2000} (OXALI), UKF-NB-3^{r}PCL^{20} (PCL), UKF-NB-3^{r}TOPO^{15} (TOPO), UKF-NB-3^{r}VCR^{10} (VCR), and UKF-NB-3^{r}VINOR^{20} (VINOR).

The following chemoresistant UKF-NB-2 sublines were derived from the RCCL collection: UKF-NB-2^{r}CDDP^{1000}, UKF-NB-2^{r}DOX^{20}, and UKF-NB-2^{r}VCR^{10}.

The following chemoresistant UKF-Rhb-1 sublines were derived from the RCCL collection: UKF-Rhb-1^{r}CDDP^{1000} and UKF-Rhb-1^{r}DOCE^{10} (DOCE), UKF-Rhb-1^{r}DOX^{10}, UKF-Rhb-1^{r}GEMCI^{10}, UKF-Rhb-1^{r}IRINO^{200}, UKF-Rhb-1^{r}MEL^{400}, UKF-Rhb-1^{r}OXALI^{1000}, and UKF-Rhb-1^{r}VCR^{10}

Moreover, the following melanoma sub-lines were derived from the RCCL collection: Colo-679^{r}VCR^{20}, Colo-679^{r}PLX4032$^{10\,\mu M}$ (PLX4032, vemurafenib), MelHO^{r}VCR^{20}, MelHO^{r}CDDP^{1000}, MelHO^{r}DAC^{20}, and MelHO^{r}PLX4032$^{10\,\mu M}$.

The corresponding IC_{50} values for the parental cells and their drug-resistant sublines are provided in Supplementary Table 4.

All cells were propagated in IMDM supplemented with 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C. Cells were routinely tested for mycoplasma contamination and authenticated by short tandem repeat profiling.

Standard molecular cloning techniques were used to generate lentiviral vectors based on Lentiviral Gene Ontology vector technology (see http://www.lentigo-vectors.de), and cell transduction was performed as described before.[11,18,34]

Viability assay. Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay after 120 h of incubation, modified as described before.[11,18]

Irradiation procedure. A total of 10^4 cells were irradiated at room temperature in 96-well cell culture plates (Greiner, Bio-ONE GmbH, Frickenhausen, Germany) with single doses of X-rays ranging from 1 to 10 Gy using a linear accelerator (SL 75/5, Elekta, Crawley, UK) with 6 MeV photons/100 cm focus–surface distance with a dose rate of 4.0 Gy/min. Sham-irradiated cultures were kept at room temperature in the X-ray control room, whereas the other samples were irradiated.

Caspase 3/7 activation. Caspase activation was measured using the Caspase-Glo 3/7 Assays (Promega, Mannheim, Germany) following the manufacturer's instructions.

Mutation analysis of p53. *TP53* gene sequencing on cDNAs was performed using the following four pairs of primers: TP53 Ex2-3-f 5-GTGA CACGCTTCCCTGGAT-3 and TP53 Ex2-3-r 5-TCATCTGGACCTGGGTCTTC-3; TP53 Ex4-5-f 5-CCCTTCCCAGAAAACCTACC-3 and TP53 Ex4-5-r 5-CTCC GTCATGTGCTGTGACT-3; TP53 EX6-7f 5-GTGCAGCTGTGGGTTGATT-3 and TP53 Ex6-7r 5-GGTGGTACAGTCAGAGCCAAC-3; and TP53 Ex8-9-f 5-CCTCA CCATCATCACACTGG-3 and TP53 Ex8-9-r 5-GTCTGGTCCTGAAGGGTGAA-3. In addition, all cell lines were examined for TP53 mutations by sequence analysis of genomic DNA as described previously.[35] PCR was performed as described before.[35] Each amplicon was sequenced bidirectionally.

Real-time PCR. Total RNA was isolated from cell cultures using TRI reagent (Sigma-Aldrich, Munich, Germany). Reverse transcription and real-time PCR were performed as described before,[36] using TaqMan Gene Expression Assays (Applied Biosystems, Darmstadt, Germany). The results are presented as fold change.

Transcriptomics. Triplicates of UKF-NB-3, UKF-NB-3^{r}Nutlin$^{10\,\mu M}$, UKF-NB-3^{r}VCR^{10} UKF-NB-3^{r}DOX^{20}, and UKF-NB-3^{r}CDDP^{1000} cells were analysed for global cellular gene expression at the mRNA level using GeneChip HGU133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) by the Fraunhofer Institut für Zelltherapie und Immunologie (Leipzig, Germany). mRNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Expression data were processed using the R/bioconductor packages '*gcrma*' and '*limma*' (http://www.r-project.org/ and http://www.bioconductor.org/) in order to detect differentially expressed genes (FDR).[37] The results were corrected for multiple testing to control the FDR using the approach of Benjamini and Hochberg.[38] Hierarchical clustering analysis was performed, and heatmaps were visualised using the R package (http://www.r-project.org).

Signal transduction pathway bioinformatics. Additional statistical analysis to interpret significant expression changes was focusing on a pathway analysis using the PANTHER database (http://www.pantherdb.org), which identifies global patterns in expression.[39] For each expert-curated pathway in the database, potential differential expression was determined by a binomial test,[40] with subsequent Bonferroni correction for multiple testing, using the PANTHER human gene reference list matching our microarrays and lists of differentially expressed genes that passed an multiple-testing corrected threshold of FDR < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)