SHORT REPORT

Potential for treatment of liposarcomas with the MDM2 antagonist Nutlin-3A

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The MDM2-antagonist Nutlin 3A can efficiently induce apoptosis in osteosarcoma cell lines with amplified *MDM2*. However, Nutlin-based therapy could be even more important in more common sarcoma types where this aberration is frequent. The well- and dedifferentiated liposarcomas have complex marker chromosomes, consistently including multiple copies of the *MDM2* locus. Since amplification seems to be a primary aberration in these tumors, whereas amplification in osteosarcoma generally is a progression marker, the underlying biological mechanisms may be different. We have therefore investigated the molecular response to Nutlin treatment in several liposarcoma cell lines with such markers, as well as a panel of other sarcoma cell lines. We report that Nutlin efficiently stabilized p53 and induced downstream p53 dependent transcription and apoptosis in liposarcoma cells with amplified *MDM2 in vitro*. Some effect of Nutlin was also observed on cell lines without amplified *MDM2* but with wt *TP53*, but no apoptosis was induced. The MDM4 protein, reported to interfere with the reactivation of p53, was undetectable in cells with amplified *MDM2*. Thus, Nutlin represents a promising new therapeutic principle for the treatment of an increasing group of sarcomas.
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The activity and level of the tumor suppressor protein p53 is negatively regulated by the E3 ligase MDM2, which controls both its ability to trans-activate downstream genes and its proteasome-mediated degradation.[1] In tumors with wild type p53, its activity may be blocked through amplification and overexpression of the *MDM2* locus.[2,3] Due to the central role of p53 in oncogenesis and therapy response, the p53-MDM2 interaction is an interesting target for small-molecular therapy. The goal is to reactivate p53 and induce downstream effects leading to programmed cell death or increased response to DNA-damaging therapies, as has been pursued by several groups.[4–9] One of the most promising MDM2 antagonists is Nutlin 3A, which has been shown to activate wild type p53 in cancer cell lines, inducing cell cycle arrest and apoptosis in various cell lines, including osteosarcomas with amplified *MDM2*.[10,11] However, amplification of *MDM2* is much more frequent in the more common subtype of well-differentiated liposarcoma (WDLS) and dedifferentiated variants, where virtually all tumors contain complex marker chromosomes that always include multiple copies of *MDM2*.[12] Since the role of MDM2 in the etiology of these tumors seems different from that in osteosarcomas, we set out to investigate the potential for Nutlin treatment and its molecular effects. This particular type of liposarcoma can be hard to eradicate and may dedifferentiate to aggressive variants with high malignancy. Since inactivation of p53 may also be achieved by amplification and overexpression of MDM4,[13–15] we also investigated whether MDM4 could be important for the response in WDLS, although we have found only low levels of amplification of *MDM4* in our sarcoma panel (Ohnstad, unpublished data).

For these investigations we chose 5 cell lines with amplified *MDM2*, including 3 WDLS-derived lines, and, since Nutlin has been reported to induce apoptosis in haematological cancers even when *MDM2* is not amplified,[4,16,17] added 5 control cell lines with normal MDM2-level, of which 3 had either no or mutated *TP53* mRNA (Table I). The cell lines with high-level amplification consistently expressed very high levels of *MDM2* mRNA (Table I), and protein (Fig. 3), whereas the cell lines with normal gene number all had low levels in comparison.

We first examined the *in vitro* response of our cell panel to the active Nutlin 3A enantiomer and, as control, the virtually inactive 3B enantiomer (Table I). All cell lines with amplified *MDM2* responded well to Nutlin 3A, with half-maximal growth inhibitory concentration (IC_{50}) from 0.6–1.9 μM, although MHM did not reach total growth inhibition under the conditions used to determine IC_{50}. In two of the control cell lines with wt *TP53* (RMS13 and U2OS) partial growth inhibition was observed (IC_{50} from 3.3–3.5 μM), whereas the lines with mutated *TP53* (SW872, KPD, SAOS2) did not respond to Nutlin ($IC_{50} > 10$ μM). We documented a similar response pattern across the cell panel for drug exposure of 24 and 72 hr. Nutlin 3B did not inhibit growth in any of the cell lines tested (data not shown).

We further investigated the transcriptional response downstream of p53 with either of the Nutlin enantiomers. Transcript levels for *BAX*, *BCL2*, *p21*, *p53*, *MDM2* and *p14^{ARF}* were assayed after 4, 24 and 48 hr of treatment (Figs. 1*a* and 1*b*). 48 hr data exist only for p21, p53 and MDM2 (supplementary Figs. II and III). As expected, cell lines with high *MDM2* copy number showed a strong and Nutlin 3A-dependent upregulation of p21 transcripts (Fig. 1*a*, unpaired *t*-test *vs.* control, $p < 0.005$) and protein (Fig. 3), indicating reactivation of p53. This was supported by increased p53 protein levels (Fig. 3) probably due to protein stabilisation.[5,10,23] However, upon treatment with 10 μM Nutlin 3A, p53 mRNA levels reproducibly decreased in all our sensitive liposarcoma cells (Fig. 1*a*, unpaired *t*-test *vs.* control $p < 0.01$), although less so with Nutlin 3B (Fig. 1*b*). This is contrary to what was observed in OSA cells (Fig. 1*b*, Refs. 10–11.). Although we cannot explain this behaviour at present, we note that in the MHM cells, originating from a parosteal osteosarcoma, also having marker chromosomes similar to those in WDLS, p53 showed the same decline, perhaps due to a similar etiology.

Consistent with p53 activation, levels of MDM2 mRNA and protein increased upon Nutlin 3A treatment (except in FU-DDLS-1; Figs. 1*a* and 3, unpaired *t*-test *vs.* control $p < 0.025$). Only moderate effects on p14^{ARF} mRNA could be observed.

TABLE I – DRUG SENSITIVITY, GENOTYPES AND CLINICAL AND BIOLOGICAL CHARACTERISTICS OF THE SARCOMA CELL LINE PANEL

Cell line	Histology	Patient sex/age	Origin	Site	TP53	MDM2 copy number	MDM2 mRNA level	IC50 (μM)	TGI (μM)
T449	WDLS	F/68	Primary	Retroperitoneum	wt	94.6 ± 6.0	77 ± 6	0.6 ± 0.1	1.9 ± 0.6
T778	WDLS	F/69	Relapse	Retroperitoneum	wt	59.8 ± 1.8	28.5 ± 1.6	1.0 ± 0.3	2.3 ± 0.9
FU-DDLS-1	DDLS	M/61	Relapse	Retroperitoneum	wt	70.6 ± 6.1	87 ± 10.9	0.6 ± 0.2	2.1 ± 1.0
OSA	OS	M/19	Primary	Femur	wt	49.1 ± 1.1	43.2 ± 9.4	0.9 ± 0.3	2.9 ± 0.4
MHM	OS	F/42	Relapse	Pelvis	wt	22 ± 1.6	59.5 ± 1.9	1.9 ± 0.3	>10
SW872	LS	M/36	Unknown	Unknown	mut	1.1 ± 0.1	0.7 ± 0	>10	>10
KPD	OS	M/8	Primary	Femur	mut	0.5 ± 0	1.1 ± 0	>10	>10
RMS13	RMS	M/17	Primary	Unknown	wt	1.9 ± 0.2	0.9 ± 0	3.5 ± 1.3	>10
SAOS2	OS	F/11	Unknown	Unknown	mut	1.4 ± 0.2	2.5 ± 0.2	>10	>10
U2OS	OS	F/15	Primary	Tibia	wt	0.7 ± 0.1	3.1 ± 0.2	3.3 ± 1.1	>10

WDLS, well differentiated liposarcoma; DDLS, dedifferentiated liposarcoma; OS, osteosarcoma; LS, undifferentiated liposarcoma, unspecified; RMS, rhabdomyosarcoma; wt, wild type; mut, mutated; TGI, total growth inhibition; IC$_{50}$, concentration required for 50% growth inhibition. The drug concentration required for 50% growth inhibition (IC50), and total growth inhibition (TGI) was determined using the Sulphorhodamine B (SRB) assay[18] after 120 h exposure to Nutlin 3A. IC$_{50}$ and TGI are presented as mean ±SD of at least 3 experiments. MDM2 copy number was determined with SYBR-green RT-PCR on 5 nanograms of genomic DNA, initially purified using a phenol-based procedure adapted from.[19] The albumin gene was used as internal normalization reference. Values were expressed relative to human genomic female DNA (Promega, Southampton, UK). Primers: MDM2 forward 5′ AAGCCAAACTGGAAAACTCAACAC 3′. MDM2 reverse 5′ CAGGAACATCAAAGCCCTCTTC 3′. Albumin forward 5′ TTTATTCACATCATTCTCTC 3′. Albumin reverse 5′GAGTGAGATATGAGTTGAG 3′. Relative MDM2 expression was determined as described in the legend of Figure 1. TP53 genotypes were determined by the Roche TP53 Genechip, as described elsewhere (Ohnstad et al., manuscript in preparation). The cell lines MHM (OS45[20]) and KPD (OS06[21]) were established from primary patient samples at the Norwegian Radium Hospital. T449 (93449) and T778 (94778) were established from primary and relapsed tumor of the same patient at Hopital de l'Archet.[12] Dr. A. Thomas Look at St Jude's hospital, Memphis, USA, kindly donated the cell lines RMS13 (Rh30) and OSA (SJSA-1 or CRL2098; also available from the ATCC). The FU-DDLS-1 cell line was kindly provided by Dr. J. Nishio, Fukuoka University, Japan.[22] SAOS2 (HTB85), U2OS (HTB96) and SW872 (HTB92) cells were purchased from the American Type Culture Collection, Rockville, MD.

In some of the MDM2-amplified lines, in particular MHM and T778, but also, e.g., for MDM2 in OSA, some weaker effects could also be observed following treatment with the 200-fold less active Nutlin 3B enantiomer.

By expression analysis, a proapoptotic change of the BAX/BCL2 transcript ratio was observed (Figs. 1a and 1b), and Nutlin 3A induced cell cycle arrest and DNA fragmentation (positive TUNEL-labeling) consistent with apoptosis after 24 hr only in cell lines with amplified MDM2 (Figs. 2a and 2b). In the wt TP53-cell lines U2OS and RMS13 where MDM2 was not amplified, we observed cell cycle arrest (Fig. 2a), but no increase of apoptotic cell fraction upon Nutlin 3A treatment (Fig. 2b, supplementary Fig. I).

Apoptosis as the mechanism of cell death was confirmed by activation of Caspase 3 (CASP3) and cleavage of Poly(ADP-Ribose)-Polymerase (PARP) in MDM2-amplified cells (Fig. 3), although a specific PARP cleavage product of 62 kDa, perhaps involving calpain,[24] could only be detected in the MHM cells.

The structurally related MDM4 protein can also inhibit p53 activity, may form heterodimers with MDM2, and can apparently diminish the Nutlin response because it is insensitive to inhibition by Nutlin.[25–27] An exception has been reported for retinoblastoma, where MDM4 is amplified but Nutlin is efficiently killing retinoblastoma cells.[28] Although MDM4 has been reported to be amplified in several cancer types,[14,29] this was generally not the case in our tumor panel (Ohnstad et al., unpublished). We nevertheless investigated the possible involvement of MDM4 in the cellular response to Nutlin in 4 of the cell lines. As can be seen in Figure 3, the 80 kDa MDM4 protein could be detected in the U2OS control line, but not in the MDM2 amplified lines, regardless of Nutlin treatment. This is most likely caused by proteasomal degradation of MDM4 mediated by the very high MDM2 levels, as previously reported.[30] Furthermore, a smaller band of ~47 kDa appeared upon treatment with Nutlin in the amplified cell lines, which may be a degradation intermediate because of some inhibitory effect of Nutlin. MDM4 isoforms of this size have been reported previously in other cell lines.[29] But the degradation mechanisms are complex, and further investigations are evidently necessary to determine the exact nature of these bands.

In contrast to e.g. osteosarcomas, the well-differentiated subtype of liposarcomas and its more malignant derivatives have wild type p53 and amplified MDM2.[12] However, little is still known of the impact of p14^{ARF} and MDM4 on p53 activity in this sarcoma subtype. In this work, we detected Nutlin-induced apoptosis only in cell lines with high MDM2 levels and wild type TP53, and with p14^{ARF} seemingly intact. This is in line with the hypothesis that tumors with amplified and overexpressed MDM2 contain an (otherwise) intact p53 pathway,[10] although contradictory findings have been described in samples with low MDM2 expression and various genotypes of MDM4, P14^{ARF} and TP53.[4,7,16,17,31,32] However, we cannot compare the absolute MDM2 levels with these studies, due to lack of data at gene, transcript and protein level. Therefore, at present, the key requirement for Nutlin sensitivity in sarcomas seems to be wild type TP53, in line with previous publications on Nutley inhibitors.[6–8,10,11,17,23] In U2OS and RMS13, representing the large fraction of sarcomas where both MDM2 and TP53 are wild type and show only low-level overexpression of the MDM2 locus, some other, as yet undetected, aberration most likely blocks the pathway downstream of p53, preventing apoptosis. Although several of such aberrations are known,[33,34] the limited selection of liposarcoma cell lines available here does not allow us to elucidate how this pathway may be deranged in such cells. More work on e.g. MDM4 must be done to clarify what separates regulation of MDM2-p53 in (lipo)sarcomas from the other classes of human cancers sensitive to MDM2 antagonists.

In conclusion, Nutlin 3A induces apoptosis efficiently in a major subset of liposarcomas, and from our data one would expect that TP53 genotype together with MDM2 amplification predicts sensitivity to this therapeutic agent. Both the mostly juvenile osteosarcomas, and adult liposarcomas, with their high mortality, are very much in need of new treatment options. Depending on the status of the p53 pathway, Nutlin could act as a sensitizer to cytotoxic therapy or protect proliferating normal cells from chemotherapy- or radiation-associated DNA-damage,[35] As proposed lately by several groups,[15,26] the lack of response to Nutlin in some tumor types suggests the design of a novel MDM4 inhibitor that may be combined with Nutlin 3A to increase the group of cancers where p53 may be reactivated. The promise of an orally administered specific and efficient drug with low toxicity makes this MDM2 inhibitor an attractive new treatment option for an increasing range of tumors. Treatment of human sarcoma-xenografts in immunodeficient mice with Nutlin 3A has already been successful,[11] and clinical trials in sarcoma patients may now be contemplated.
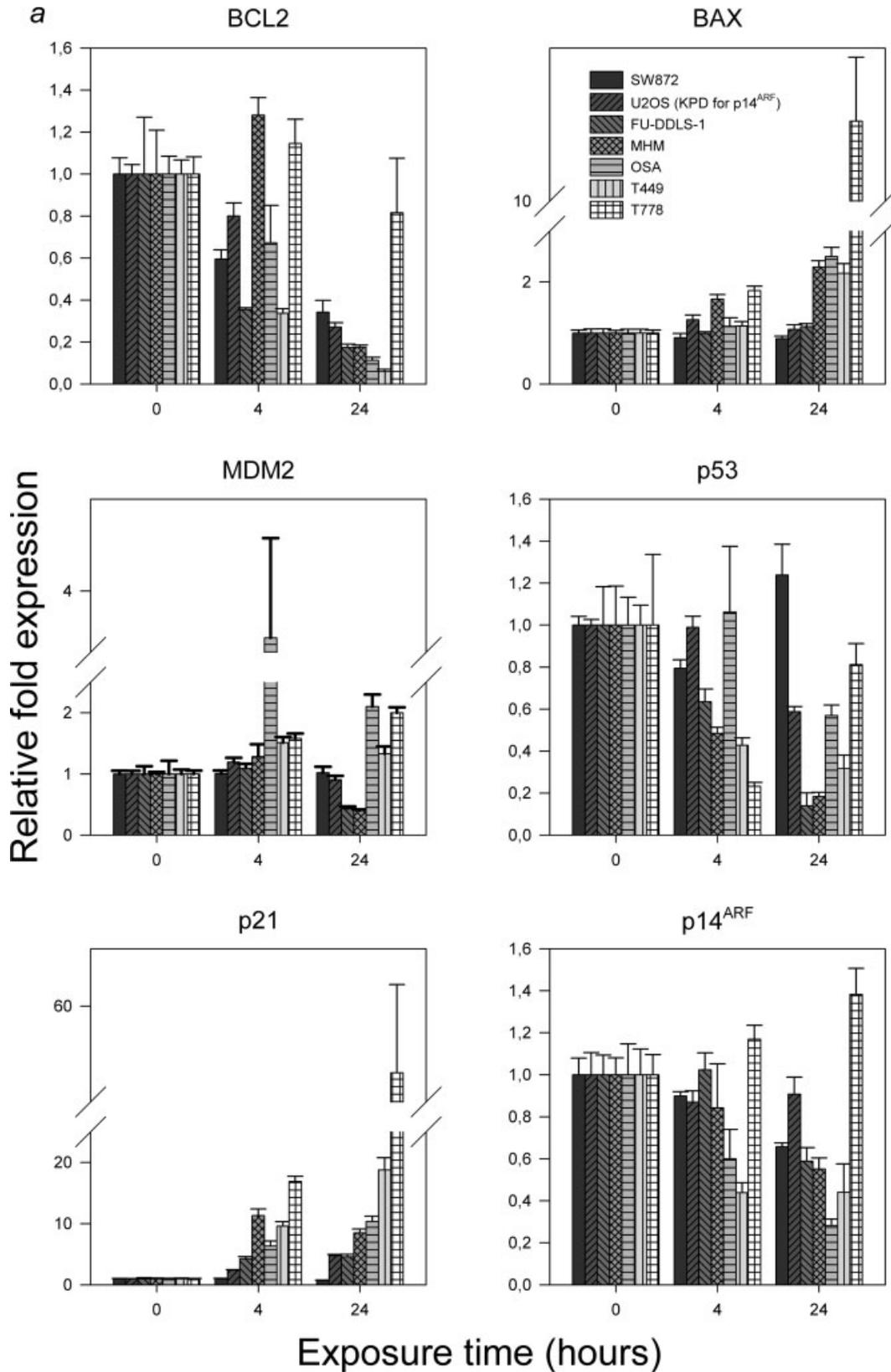
FIGURE 1 – Expression of selected genes in a cell line panel after treatment with (*a*) 10 μM Nutlin 3A or (*b*) 10 μM Nutlin 3B. Data are presented as fold-change expressed relative to untreated cell line control ($t = 0$) ±SD of at least 3 experiments. Three of the noninformative control cell lines were omitted to simplify the figure. All RT-PCR analyses were performed using Applied Biosystems 7500 thermal cycler and software. Briefly, total RNA was isolated using Trizol (Invitrogen, Paisley, UK) following the manufacturer's protocol. 2–5 ng cDNA was amplified, and standard curves generated using universal microarray reference RNA (UMR, Stratagene, La Jolla, CA). As internal normalization reference we used VIC-labeled beta-2-microglobulin, *B2M*. The NanoDrop apparatus (NanoDrop Technologies, Wilmington, DE) and the 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) were used to assay RNA/DNA quality and purity. (TaqMan assay IDs are listed in supplementary Table I). The legend is uniform for 5 of 6 panels except as indicated for p14^{ARF}, where KPD results were plotted instead of U2OS.
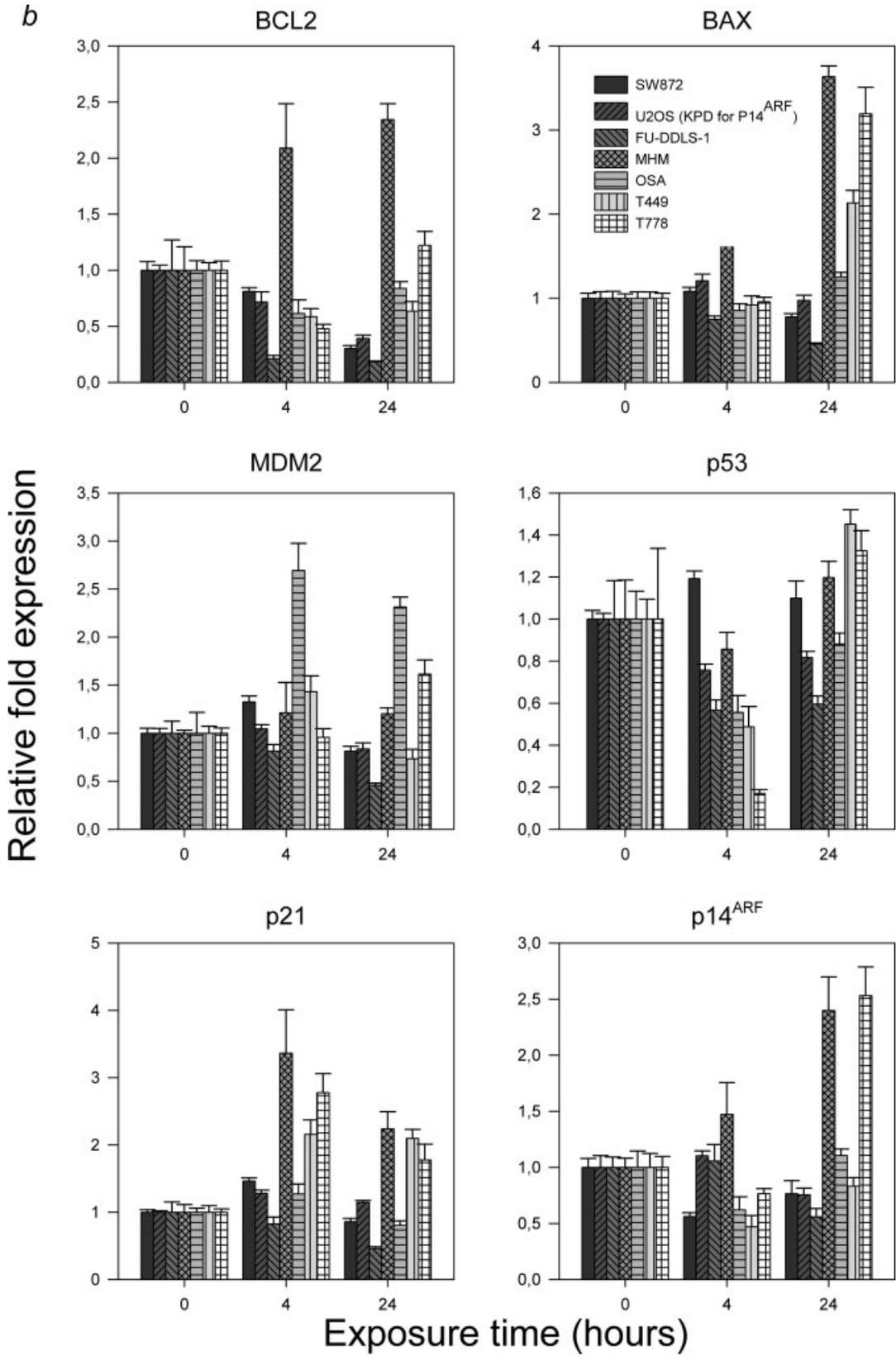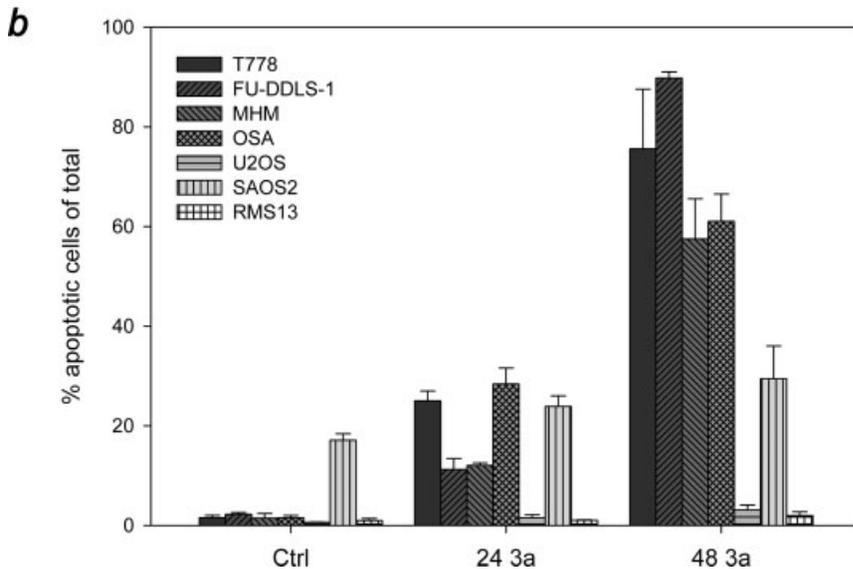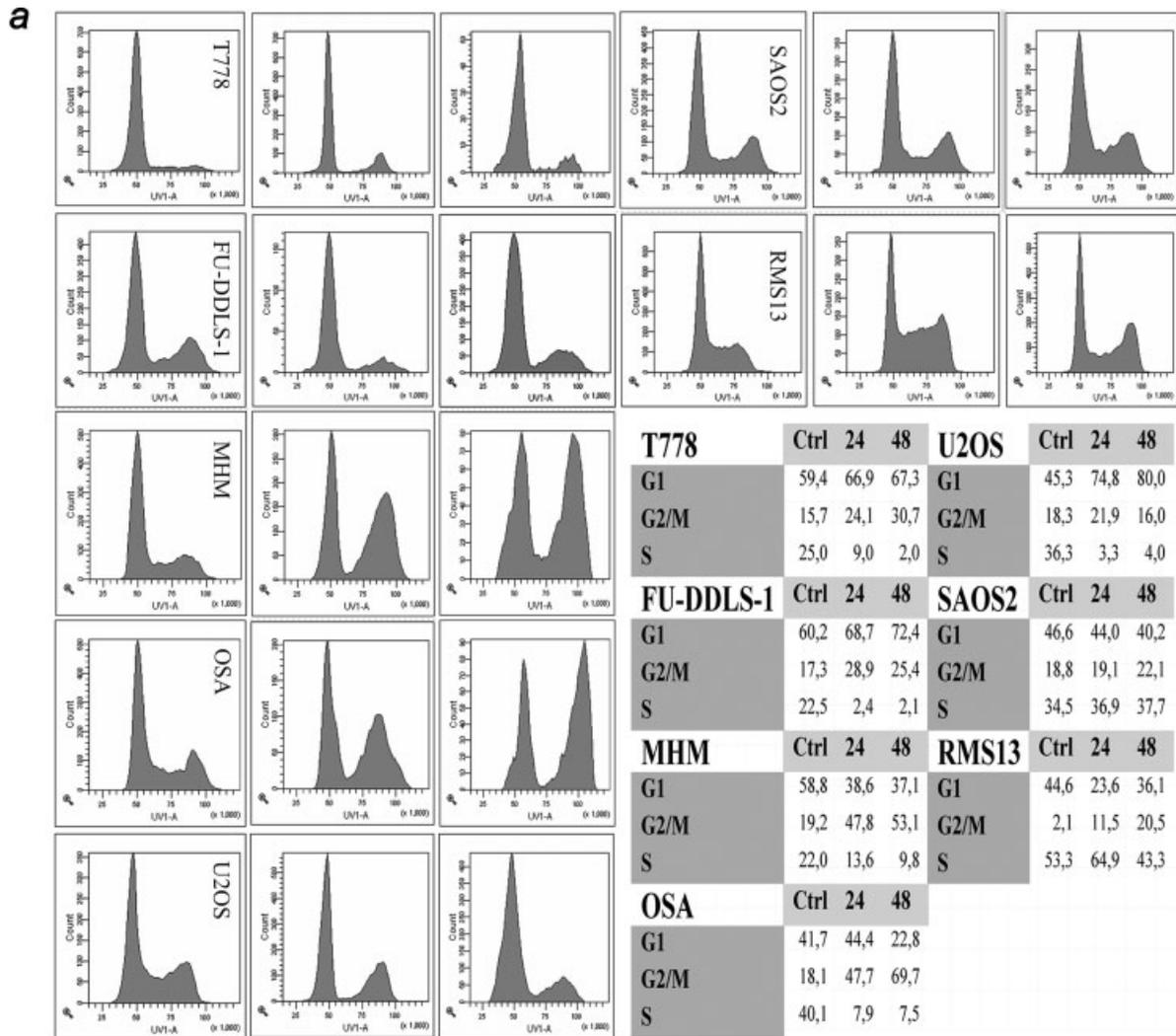
FIGURE 1 – (CONTINUED)

FIGURE 2 – Apoptotic response of seven selected sarcoma cell lines to treatment with 10 μM Nutlin 3A for 24 and 48 hr compared to untreated controls. Data represent the mean of minimum 2 experiments. 10,000 cells were counted in each run. (*a*) Cytograms and cell cycle-distributions in viable cells, expressed as percentage of total population, rounded to the full percentage value. (*b*) Apoptotic percentage of total, presented with standard deviation. Apoptotic cells were quantified after methanol-fixation by TUNEL labeling (Gavrieli *et al*., 1992) using a kit (#333574) from Hoffmann-La Roche, NJ, USA. The biotin-labeled cleavage sites were labeled with FITC-conjugated streptavidin (RPN1232VI, Amersham Biosciences, Little Chalfont, England). Two μg/ml Hoechst 33258 (Sigma-Aldrich, St. Louis, MD) was used to stain genomic DNA. Cells were analyzed with the ModFit and Becton Dickinson FACS DiVa software and flow cytometer with Argon (488 nm) and Krypton UV lasers (356 nm).
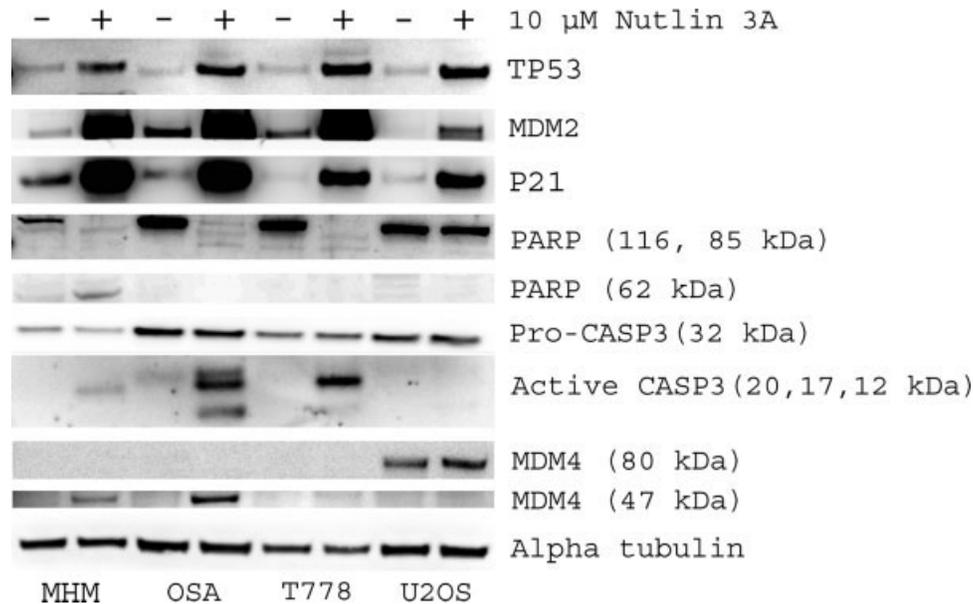
FIGURE 3 – Activation of the p53 pathway in selected cell lines after 10 μM Nutlin 3A-treatment for 26 hours. Complete cleavage of PARP and activation of CASP3 indicates apoptotic activity in cell lines with *MDM2* amplification. Data are representative of one of three separate experiments. Total protein was obtained with lysis buffer from a caspase assay kit (BioSource, Camarillo, CA), and concentration was measured using the BCA (bicinchoninic acid) method (kit #23225, Pierce, Rockford, IL). 25 μg of lysate was separated on 4–12% NuPage Bis-Tris gradient gels (Invitrogen, Paisley, UK) and transferred to PVDF membranes (Millipore, Bedford, MA) in blotting buffer with 20% (v/v) methanol, using wet blot equipment (BIO-RAD, Hercules, CA). Bands were visualized using SuperSignal West Dura ECL (#34076, Pierce, Rockford, UK) and the Kodak IR2000 Imager and software (Eastman Kodak, Rochester, NY). Antibodies (monoclonal except when noted): anti-PARP (1:2,000, Calbiochem 512739/Merck, Darmstadt, Germany), anti-CASP3 (1:2,000, R&D Systems AF-605-NA, Abingdon, UK), polyclonal anti-HDMX/4 (1:20,000, Bethyl Laboratories BL1258, Montgomery, TX), anti-p53 (1:3,000 Santa Cruz sc-6243, Biotechnology, Santa Cruz, CA), anti-MDM2 (1:200, Chemicon MAB1434, Temecula, CA), anti-tubulin (1:200, Calbiochem CP06/Merck, Darmstadt, Germany), polyclonal anti-p21 (1:250, Santa Cruz sc-397, Biotechnology, Santa Cruz, CA). All HRP-conjugated secondary antibodies were from DAKO, Glostrup, Denmark.

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