Mithramycin SK modulates polyplody and cell death in colon carcinoma cells

Marc Bataller,1 Carmen Méndez,2 José A. Salas,2 and José Portugal1

1Instituto de Biología Molecular de Barcelona, CSIC, Parc Científic de Barcelona, Barcelona, Spain and 2Departamento de Biología Funcional-Instituto Universitario de Oncología del Principado de Asturias, Universidad de Oviedo, Oviedo, Spain

Abstract

During a normal cell cycle, polyplody and aneuploidy can be prevented by several checkpoints, which are mainly p53 dependent. Here, we show that treatment of HCT-116 (p53+/+) colon carcinoma cells with the novel antitumor antibiotic mithramycin SK (MSK) results in polyplody and mitotic catastrophe, which occurs after a transient halt in G1 phase followed by the overtaking of the G2-M checkpoint when treated cells are incubated in a fresh drug-free medium. Cells reentering aberrant mitosis mainly died by necrosis, although active caspase-3 was observed. Our results indicate that a decrease in p53 RNA and protein levels, together with concomitant changes in the expression of other proteins such as p21WAF1, were involved in MSK-induced polyplody. Furthermore, the effects of MSK on HCT-116 (p53+/+) cells cannot be attributed exclusively to the down-regulation of p53 by MSK, because these effects differed from those observed in MSK-treated HCT-116 (p53−/−) cells. The p53−/− cells died mainly from G2-M arrested in mitosis in response to spindle damage, but after the result of aberrant mitosis, which can induce polyploidy (1, 6) and the occurrence of large multinucleated cells and micronucleation (1, 5). In several cell types, mitotic catastrophe can be accompanied by the activation of caspases, which resembles apoptotic cell death (7–9).

Cancer cells often present altered ploidy (10). In addition to changes in the total number of chromosomes, these cells are aneuploid because they usually contain a variety of other gross chromosomal rearrangements, deletions, and nonreciprocal translocations (10). Cells entering mitosis after DNA damage may be confronted by the activation of a tetraploidy checkpoint that results in G1 arrest (10), which can eventually be followed by apoptosis (11). However, the presence of this checkpoint in mammalian cells has been challenged (12). Furthermore, if a hypothetical tetraploidy checkpoint were compromised (e.g., in p53-defective cells), tetraploid cells might proceed through cell division and reenter mitosis, thereby undergoing further polypliodization. This would often result in aneuploidy and cell death, yet viable clones could still emerge (13).

Treatment with spindle-damaging agents leads to transient mitotic arrest as a result of the activation of the spindle checkpoint, which is followed by mitotic slippage, and the anomalous exit from mitosis without sister chromatid segregation or cytokinesis (13). However, the resulting tetraploid cells are arrested in G1, thereby preventing entry into the S phase and polypliodization (14). Cells lacking either p53, p21WAF1, or pRb are normally arrested in mitosis in response to spindle damage, but after mitotic slippage these cells enter the S phase and endoreplicate their DNA, which results in polypliodization despite their intact spindle checkpoint. This finding indicates strict dependence of the postmitotic G1 checkpoint on the p53 pathway. The activation of p53 protects healthy cells from polypliodization (10). Nevertheless, little is known about the molecular mechanisms that cause p53 activation in response to mitotic failure. There is a functional interplay between the mitotic spindle

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Requests for reprints: José Portugal, Instituto de Biología Molecular de Barcelona, CSIC, Parc Científic de Barcelona, Baldirí Reixach, 10, E-08028 Barcelona, Spain. Phone: 34-93-403-4959; Fax: 34-93-403-4979. E-mail: jmbmcm@ibmb.csic.es

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The aureolic acid antibiotic mithramycin A (MTA), also known as plicamycin, has been used for the treatment of Paget’s disease and testicular carcinoma; however, its current clinical use is limited by severe toxicity (2). The secondary metabolite of MTA, has been generated by insertional inactivation of the gene mtmW in Streptomyces argillaceus (26). MSK and MTA contain an identical tricyclic chromophore with a unique hydrophilic side chain attached in 3-position and distinct saccharide chains attached in positions 2 and 6. MSK has been tested in vitro against several human cancer cell lines and shows an improved therapeutic index compared with MTA (26) in addition to inhibiting transcription of several genes (29).

The availability of new mithramycin analogues with improved pharmacologic properties, together with a better understanding of their effects on gene expression, opens up new perspectives for the therapeutic use of this class of compounds, which have gained renewed attention as therapeutic agents in cancer- and non-cancer-related diseases (30, 31).

We observed that HCT-116 (p53+/+) colon carcinoma cells were arrested in G2 after treatment with MSK. However, after withdrawal of the drug, cells growing in fresh drug-free medium reentered the cell cycle and underwent polyploidization after entering mitosis. Giant cells containing multinuclei and micronuclei were observed before most of the cells were committed to cell death by necrosis, yet caspase-3 activity was observed. In contrast, MSK-treated p53−/− cells died mainly from G2-M through early p53-independent apoptosis, which can be followed by secondary necrosis.

**Figure 1.** MSK is more cytotoxic than MTA in HCT-116 colon carcinoma cells, and it alters cell cycle distribution. A, chemical formula of MSK. B, flow cytometry analyses of HCT-116 colon carcinoma cells treated with the IC50 doses for p53+/+ and p53−/− cells determined after 72-h continuous treatments (Supplementary Table S1). Experiments labeled “72 + 24 h” and “72 + 72 h” correspond to continuous treatments for 72 h followed by 24 or 72 h incubation in fresh drug-free medium after withdrawal of the drug.

**Materials and Methods**

**Materials**

MTA and its secondary metabolite MSK were isolated and purified from the producing organisms as described previously (25, 26). Stocks of MTA or MSK were prepared as 1 mmol/L solutions in sterile 150 mmol/L NaCl, maintained at -20 °C, and brought to the final concentration just before use.

**Cell Culture and Drug Treatments**

HCT-116 (p53+/+) human colon carcinoma cells and its derivative p53−/− were kindly provided by Dr. B. Vogelstein (Johns Hopkins University). Cells were maintained in 50% Dulbecco’s MEM (Life Technologies)/50% Ham’s F-12 medium (Cambrex), supplemented with 10% fetal bovine serum (Life Technologies), 100 units/mL penicillin, and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO2. Exponentially growing cells subcultured at a density of 2.5 × 104/mL were incubated with various concentrations of MTA or MSK at 37 °C for times indicated in the legend to figures.

**Cytotoxicity Assays**

The effect of MTA or MSK on HCT-116 cell growth was determined using the MTT method as described elsewhere (32). In brief, cells subcultured at a density of 2.5 × 104/mL were incubated with various concentrations...
of MTA or MSK at 37°C for 72 h. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (Sigma) was added to each culture.

**Cell Cycle Analysis and Flow Cytometry**

After treatment with either MTA or MSK for various periods, the cells were harvested, fixed with 70% ethanol, and stained with propidium iodide (PI; Sigma) as described elsewhere (32). Nuclei were analyzed with a Coulter Epics-XL flow cytometer using the 488 nm line of an argon laser and standard optical emission filters.

To analyze the mitotic fraction, fixed cells were incubated with the anti-phospho-histone H3 (Ser10) antibody (Upstate) followed by Cy2-conjugated secondary antibody (Jackson ImmunoResearch). Stained cells were then counterstained with PI and analyzed for Cy2 and PI fluorescence by flow cytometry.

**Determination of DNA Synthesis**

DNA synthesis in MSK-treated cells was assayed by the incorporation of 5-bromo-2′-deoxyuridine (BrdUrd; Roche Diagnostics) using a fluorescence-conjugated antibody against BrdUrd (BD Biosciences) and costained with PI.

**Immunofluorescence Staining**

HCT-116 cells treated with MSK were fixed with 4% paraformaldehyde, stained with DAPI, and inspected under an Eclipse E-800 fluorescence microscope (Nikon). To observe the mitotic spindle, cells were incubated with anti-β-tubulin antibody (Chemicon International) revealed by anti-mouse Cy3 conjugate (Jackson ImmunoResearch).

The presence of active caspase-3 was determined by using Casp-3a antibody (Cell Signaling Technology) revealed by Cy3-conjugated secondary antibody (Jackson ImmunoResearch) and analyzed under an Eclipse E-800 fluorescence microscope (Nikon).

**Assignment of Senescence-Like Growth Arrest and Cell Division**

Senescence-like growth arrest was determined, using phase-contrast microscopy, based on the percentage of senescence-associated β-Gal (SA-β-Gal) cells (33), using 5-bromo-4-chloro-3-indolyl β-d-galactosidase at pH 6.0.

**Assessment of Apoptosis and Necrosis**

Apoptosis was distinguished from apoptosis by using the Annexin V-Fluos staining kit (Roche Diagnostics) and flow cytometry in a Coulter Epics-XL flow cytometer (Coulter). Necrotic cells were characterized as two populations: Annexin V-Fluos negative/PI positive (primary necrotic cells) and Annexin V-Fluos positive/PI positive (apoptotic cells or primary necrosis).

The presence of active caspase-3 and caspase-2 was assayed together with changes in mitochondrial membrane potential (ΔΨm). Cleaved (active) caspase-3 was detected by both fluorescence staining of cells and immunoblotting (described in previous paragraphs), whereas caspase-2 was detected by using the Caspase-2/ICH-1 Colorimetric Protease Assay kit (MBL) as described elsewhere (7). To measure the changes in ΔΨm, untreated and MSK-treated cells were stained with the MitoProbe DilIC (5) Assay kit (Molecular Probes) following the supplier’s protocol. Cells were visualized by flow cytometry together with staining with PI to discriminate between living and death MSK-treated cells.

**Quantitative Real-time PCR**

Total RNA was isolated from untreated control cells and cells treated with the IC75 for MSK using the Ultraspec RNA isolation reagent (Biotecx) following the vendor’s guidelines. RNA samples were treated with RNase-free DNase I (Roche Diagnostics) and phenol-extracted.

Quantitative real-time PCR was used to analyze changes in gene expression after 4-h treatments, or 4 h after that the cells were allowed to grow in fresh drug-free medium, following a 72-h continuous treatment with MSK. Total RNA (100 µg, determined spectrophotometrically) was copied to cDNA using the Omniscript RT kit (Qagen). The appearance of amplified fragments in real time was monitored by the SYBR Green method in an ABI-Prism 7000 Sequence Detection System (Applied Biosystems) using the primers indicated in Supplementary Table S2.3 Amplification of the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene was done in parallel to allow normalization among samples. Quantitative real-time PCR conditions included an initial denaturation step at 95°C for 10 min followed by 40 cycles of a denaturation step at 95°C for 15 s and annealing/extension step at 60°C for 1 min. Results are expressed as folds of change in RNA expression of each gene, corrected by the glyceraldehyde-3-phosphate dehydrogenase gene expression.

**Western Analysis of Protein Levels**

Protein was extracted from MSK-treated and control cells, with a lysis buffer consisting of 50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40, and 0.1 mmol/L phenylmethylsulfonyl fluoride, containing 2 µg/mL aprotinin and 0.1 µg/mL leupeptin. Total protein was quantified by the Bradford assay (Bio-Rad). Denatured proteins (~30 µg) were subjected to electrophoresis on SDS-polyacrylamide gels, blotted onto Optitran BA-S85 membranes (Schleicher & Schuell), probed with the specific antibodies (see Figs. 4 and 5 legends), incubated with secondary antibodies (Jackson ImmunoResearch), and detected by chemiluminescence using Luminol (Sigma).

**Clonogenic Assay**

To study the final fate of HCT-116 (p53+/+) cells treated with the IC75 for MSK, cells were stained, after treatment for 72 h, followed by 72 h in drug-free medium, with Hoechst 33422 (Sigma) and analyzed by cytometric sorting of polyploid cells using a MoFlo flow cytometer (DakoCytomation). Optical alignment was based on optimized signal from 10 µm fluorescent beads (Flowcheck; Coulter). Purification of individual polyploid cells using the MoFlo flow cytometer was based in a droplet formation system. Sorting region (>4N) was defined on a Hoescht 33422 integral histogram, and sorting was carried out using a

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3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
200 μm tip at 5 psi. Polyploid cells were seeded, using the 200 μm tip, into separate wells in a 96-well microtiter plate (Corning Coulter) in fresh drug-free medium. The presence/absence of cell duplication, or endoreplication without cytokinesis, was observed directly under the microscope up to 14 days after sorting.

Results

Comparison of the Antiproliferative Effects of MTA and MSK on HCT-116 Colon Carcinoma Cells

To establish the experimental conditions to analyze the effects of MTA and MSK on HCT-116 (p53+/+) or HCT-116 (p53−/−) colon carcinoma cells, we determined

Figure 2. MSK-treated HCT-116 (p53−/−) cells undergo mitotic catastrophe and some of them become polyploid. A, representative fluorescence microscopy images (DAPI staining) showing examples of the effects of MSK. After 72-h continuous treatment, cells were allowed to grow in drug-free medium and they became larger (bottom), showing diverse aberrant mitotic figures; costaining with a fluorescent antibody against β-tubulin shows the presence of multipolar mitosis (left-bottom, arrows). Most of the untreated cells were halt in G1, although some normal mitoses were also observed (top). Bar, 20 μm. B, assay for the simultaneous detection of diploid and tetraploid cells by PI and cells in mitosis by an antibody against H3Sp10 (specific marker Ser-10-phosphorylated histone H3). The percentage of mitotic cells is indicated on the plots. Cells that were allowed to grow for 48 h after the withdrawal of the drug (IC75 MSK, 72 + 48 h) showed not only an increase in the percentage of mitosis but also in the number of polyploid cells. C, HCT-116 (p53−/−) cells stained with a fluorescein-conjugated antibody against BrdUrd to detect those incorporating BrdUrd (cells in S phase) together with PI to assess cell cycle distribution. After treatment with MSK, DNA synthesis occurred in polyploid cells undergoing endoreplication. D, MSK-treated p53−/− cells did not incorporate BrdUrd; thus, they were not duplicating their DNA because BrdUrd labels only those cells that have progressed into or through S phase (cf. C and D).
the cytotoxicity of these two molecules after continuous treatments for 72 h. The IC$_{50}$ and IC$_{75}$ (drug concentrations required to inhibit cell growth by 50% and 75%, respectively) values indicated that MSK was more cytotoxic than MTA against these cells (Supplementary Table S1). Therefore, we focused our experiments on MSK because it shows cytotoxic effects at lower doses. MTA binds more strongly to DNA than MSK in vitro (21); thus, the greater activity of MSK may be due to its improved cellular uptake (29) or to greater specificity in its cell targeting.

**MSK Induces Polyploidization and Mitotic Catastrophe in HCT-116 (p53$^{-/-}$) Cells**

HCT-116 (p53$^{-/-}$) cells treated continuously for 72 h were halved mostly in the G$_1$ phase of the cell cycle, whereas 48.8% of p53$^{-/-}$ cells appeared to stop mainly in the S phase (Fig. 1B). To gain further insight into the accumulation of p53$^{-/-}$ cells in G$_1$ after 72 h of treatment with this compound before cycling, we used two approaches to analyze whether these cells displaying a senescence-like phenotype were senescent after 72 h of continuous treatment. Approximately 55% of HCT-116 (p53$^{-/-}$) cells showed a senescence-like phenotype (Supplementary Fig. S1). Senescence induction by MSK in these cells was transient and maintained by the presence of the drug. After the withdrawal of MSK, cells entered mitosis and underwent polyploidy, especially 8N cells, after 24 h in fresh drug-free medium (Supplementary Fig. S1C). Conversely, HCT-116 (p53$^{-/-}$) were not senescent (Supplementary Fig. S1A) and accumulated in S phase after withdrawal of MSK (Fig. 1), although these cells did not synthesize DNA, as described below. The S-like accumulation in p53$^{-/-}$ cells could be explained by DNA degradation from G$_2$-M. After 72 h in fresh drug-free medium all the MSK-treated p53$^{-/-}$ cells were dead.

HCT-116 (p53$^{+/+}$) cells displayed normal morphology 24 h after withdrawal of MSK (Fig. 2A). However, after removal of the drug, these cells entered mitosis and showed aberrant mitotic figures after approximately 48 h of growth in fresh drug-free medium and beyond (Fig. 2A, bottom row). We observed multipolar attachment of microtubules and a high percentage of large cells containing mitotic figures that showed other abnormalities, including multipolar anaphases and uneven chromosome distribution (Fig. 2A).

Furthermore, we did a two-dimensional assay to distinguish between cells that were in G$_2$ or M (Fig. 2B). The presence of cells in mitosis during and after treatment with MSK was documented using the mitotic specific marker Ser$^{10}$-phosphorylated histone H3 together with PI staining of DNA. About 4% of control (untreated) HCT-116 (p53$^{+/+}$) cells were in G$_2$-M without polyploidy (Fig. 2B), whereas treated cells showed polyploidy (>4N cells). There was an increase in both polyploidy and cells entering mitosis from G$_2$ after withdrawal of MSK. Although polyploidy was detected in fresh drug-free medium, 24 h after withdrawal of the drug (Supplementary Fig. S1C), the presence of the mitotic marker Ser$^{10}$-phosphorylated histone H3 in cells with >4N content was evident only after 48 h (Fig. 2B, bottom). Consistent with these results, untreated cells assayed for DNA synthesis by BrdUrd labeling were distributed in G$_1$, S, and G$_2$-M phases of the cell cycle, whereas cells treated with MSK were also observed as 8N cells, and a S-phase corresponding to DNA synthesis in the polyploid population was observed (Fig. 2C).

The effect of a continuous 72-h treatment with MSK on HCT-116 (p53$^{-/-}$) was clearly differed because 48.8% of the cells were in S, whereas 30.4% were in G$_2$-M rather than in G$_1$ and committed to die from G$_2$-M without apparently entering faulty mitosis. Flow cytometry showed that cells in the S phase corresponded to dead cells with DNA content higher than 2N (Fig. 1B), thus showing that they were not synthesizing DNA (Fig. 2D).

**MSK Induces Transcriptional Changes in HCT-116 Cells**

MTA has been described as an inhibitor of the interaction between some transcription factors and gene promoters (22, 34). Therefore, we sought to gain insights into whether the effects we observed for MSK were also related to a direct effect on the transcription of various key genes involved in checkpoint control and proliferation.
Quantitative RT-PCR experiments revealed the presence of significant changes in the transcription of several genes. Although after a short incubation of HCT-116 cells with MSK (4 h) the transcription of *c-myc*, *cyclin A*, and *p21WAF1* was significantly enhanced by the treatment (*P* < 0.05; Fig. 3A), this effect was not observed on *p53* and *Chk1*. The transcription of all these genes, except *p21WAF1*, was inhibited significantly (*P* < 0.01) after 72-h treatments with MSK followed by a 4-h incubation in drug-free medium (Fig. 3B). In contrast, the transcription of *p21WAF1* was enhanced compared with untreated control cells, an observation that is consistent with the transient halt of cells in G1 as shown by flow cytometry (Fig. 1B).

**HCT-116 (p53+/+) Cells Treated with MSK Die by Necrosis Rather Than by Apoptosis**

Figure 4 shows a two-dimensional assay that distinguishes apoptosis from necrosis by using Annexin V-Fluos and PI. HCT-116 (p53+/+) cells treated with MSK for

**Figure 4.** HCT-116 (p53+/+) colon carcinoma cells were committed to die by necrosis rather than apoptosis even in presence of active caspase 3; whereas p53−/− cells died by apoptosis mediated by caspase 2. **A**, flow cytometry of HCT-116 (p53+/+) cells stained with Annexin V-Fluos and PI reveals the absence of Annexin V-Fluos positive/PI negative staining of cells (apoptosis), which does not depend on whether cells were allowed to grow in fresh medium after withdrawal of the drug (top row) or they were treated continuously with MSK for 96 h (middle row). The presence of high staining with both Annexin V-Fluos and PI would correspond to leaking necrotic cells and not to primary apoptotic cells that should be not permeable to PI. However, in p53−/− cells (bottom row), apoptotic cells were observed (Annexin V-Fluos positive/PI negative). **B**, immunofluorescence microscopy with antibodies against the active form of caspase-3, counterstained with DAPI, shows the presence of large cell nuclei with multipolar mitotic spindle. Nevertheless, these MSK-treated p53+/+ cells were committed to die by necrosis rather than apoptosis (cf. **B** and **A**). Bar, 20 μm. **C**, a representative Western blot analysis of the presence/absence of active caspase-3 in MSK-treated HCT-116 cells, showing that caspase-3 was activated in p53+/+ cells after treatment, whereas it was not in p53−/− cells. The specific antibody against cleaved (active) caspase-3 was purchased from Cell Signaling Technology. **D**, ΔΨm measurements in control and MSK-treated cells stained with the DiIC1 (5) potentiometric dye (Molecular Probes). MSK did not produce a significant disruption of the ΔΨm in p53+/+ cells but a significant decrease (*, *P* < 0.05) in membrane potential was observed in MSK-treated p53−/− cells (open columns). Plot also displays the differences in PI staining (gray columns) between control and MSK-treated cells used to correct the ΔΨm data for the presence of cell death after drug treatment. Mean ± SD of three independent experiments.
Mithramycin SK Modulates Polyploidy in Colon Carcinoma

72 h were allowed to grow in fresh medium after removal of the drug. Cells were dying by necrosis (high staining with PI), whereas there was no presence of apoptotic cells (Annexin V-Fluos positive). A direct morphologic analysis, made in parallel, using double fluorescence staining consisting of DAPI and a specific antibody for active caspase-3, showed that the large cells contained multipolar mitotic figures (Fig. 4B) and some of these cells were multinucleated, in keeping with the rise of mitotic catastrophe. These mitotic figures were also observed in cells showing caspase-3 staining, yet the cytometry results indicated that these cells did not undergo apoptosis (absence of Annexin V-Fluos staining; Fig. 4). The presence of active forms of caspase-3 in p53+/+ cells dying by “primary” necrosis (Annexin V-Fluos negative/PI positive) was also confirmed by Western blot (Fig. 4C).

Direct analysis of changes in \( \Delta \Psi_m \) indicated that treatment of HCT-116 (p53+/+) cells with MSK did not produce a significant disruption of the membrane potential (Fig. 4D), whereas a significant (\( P < 0.05 \)), yet moderate, decrease in this potential was observed in MSK-treated p53−/− cells (Fig. 4D). In HCT-116 (p53−/−) cells, MSK induced a primary apoptotic pathway from G2-M, which was mediated by caspase-2 (Supplementary Fig. S2). Some apoptotic p53−/− cells eventually showed a secondary (Annexin V-Fluos positive/PI positive) necrotic phenotype (Fig. 2A). Moreover, the p53−/− cells died from G2-M arrest, apparently without entering mitosis, as the mitotic marker Ser10-phosphorylated histone H3 was not observed (not shown).

Changes in Protein Levels Might Explain the Fate of HCT-116 Cells after Treatment with MSK

Protein levels in p53+/+ cells were analyzed by Western blot to check whether changes in RNA levels (Fig. 3) were followed by a decrease in the corresponding protein. Moreover, we also examined protein levels in p53−/− cells to check for changes that may explain their peculiar behavior after treatment with equitoxic concentrations of MSK.

Figure 5 shows time-dependent changes in p21\(^{WAF1}\), p53, and cyclin B, whereas those corresponding to the active form of caspase-3 were described above because of their direct relevance in cell death mechanisms (Fig. 4C). A quantification of the changes in protein levels is shown as Supplementary Fig. S3. In p53+/+ cells, p53 levels increased during MSK treatment up to 72 h, but decreased after removal of the drug (Fig. 5A), which may facilitate polyploidy (13). After withdrawal of the drug, the presence of p21\(^{WAF1}\) was also slightly reduced (Fig. 5A), which was by the time cells were arrested in G1, and after a 72-h incubation in drug-free medium, this protein was undetectable. Meanwhile, cyclin B levels increased after removal of the drug, which is consistent with the entry of HCT-116 (p53+/+) into mitosis. In p53−/− cells, a 72-h treatment with 25 nmol/L MSK (its IC\(_{75}\)) reduced the levels of c-myc (Fig. 5B). However, after withdrawal of the drug, cells growing in fresh drug-free medium synthesized the protein to the same extent as untreated controls (Fig. 5B). Interestingly, the time-dependent changes induced by MSK in c-myc and p21\(^{WAF1}\) levels were in opposite directions: one increased, whereas the other decreased (cf. Fig. 5A and B), in concordance with that c-myc would negatively regulate p21\(^{WAF1}\).

In HCT-116 (p53+/+) cells, p21\(^{WAF1}\) decreased after withdrawal of the drug (“72 + 24 h” track in Fig. 5C) and there was also a small change in cyclin B protein levels, in line with a transient halt in G2-M after 72 h of continuous treatment with MSK, before cells entered apoptosis (see above). The p53 protein was not detected (Fig. 5C), as expected in this p53-mutant cell line (17).

A Clonogenic Analysis of Polyploid Cells Induced by MSK Reveals That Several Multinucleated Cells Survive in the Absence of Cytokinesis

Sorted (individual) HCT-116 (p53+/+) polyploid cells obtained after a 72-h continuous treatment with MSK, followed by 72 h in drug-free medium, were analyzed for clonogenic capacity, after being seeded as individual polyploid cells, for up to 14 days in fresh medium. Of these clones, 75.6% were committed to die in the following days (Fig. 6A). However, the surviving cells showed an increase in size and were multinucleated (Fig. 6B),
required for efficient postmitotic G1 arrest (13). MSK mediated p53 accumulation and a prolonged mitotic arrest are consistent with previous observations that both p53 levels was observed in HCT-116 (p53+/+) cells. These results are consistent with previous observations that both p53 accumulation and a prolonged mitotic arrest are required for efficient postmitotic G1 arrest (13). MSK appeared to “facilitate” the reentry of cells into mitosis, thus causing mitotic catastrophe. However, HCT-116 (p53−/−) cells, which are defective in checkpoint controls (17), died from G2-M after treatment with MSK by p53-independent apoptosis (36). This finding is in keeping with the observation that p53 deletion in HCT-116 cells leads to greater apoptosis induced by some, but not all, antitumor drugs (18).

Regardless of the presence of active caspase-3, HCT-116 (p53+/+) cells treated with MSK were chiefly committed to cell death by necrosis rather than apoptosis. Here, we show massive necrosis in cells bearing wild-type p53 and active caspase-3. It has been suggested that, when mitotic catastrophe occurs after DNA damage, cells are most likely to undergo slippage and multinucleation followed by nonapoptotic cell death (35), because apoptosis-prone cells, bearing damaged DNA, die before entering mitosis. Both caspase-dependent and caspase-independent mechanisms of cell death after mitotic catastrophe have been reported (7, 8, 37–39), which may end in either necrosis or apoptosis (3, 5). The mode of cell death would be determined by the cell context. The down-regulation of p53 by MSK and the large cell size, which were the result of damaged cells reentering mitosis together with the absence of cytokinesis, might produce cells that die during the abrupt onset of a general malfunction, including permeabilization of cell membranes. This notion was substantiated by the high uptake of PI in the presence of only 3% staining with Annexin V-Fluos. Our results suggest that the rate of necrosis is higher than that of apoptosis, which induces MSK-treated HCT-116 (p53+/+) cells to undergo necrosis even in the presence of active caspase-3, whereas in p53−/− cells apoptosis was mediated by caspase-2. When treated with MSK, HCT-116 (p53+/+) cells did not exhibit significant ΔΨm dissipation (Fig. 4D), which is in line with the absence of apoptosis in these cells. In contrast, the homologous p53−/− cells showed a slight, although significant, decrease in mitochondrial potential, in accordance with a previous observation that tetraploid cells treated with the DNA-damaging agent cisplatin manifest less pronounced ΔΨm dissipation (11).

When p53 is activated in response to some cell cycle errors (1, 10), proapoptotic pathways can also be activated. In contrast, we have shown that the inhibition of p53 expression by MSK results in the generation of a polyploid state, whereas the enhancement of p21WAF1 expression contributes to this state (Fig. 3). This observation agrees with the finding that p21WAF1 is not only a mediator of p53 tumor suppression (40) but also a negative regulator of p53 stability (41).

Figure 6. Clonogenic assay of polyploid cells (>4N) obtained after treatment of HCT-116 (p53+/+) colon carcinoma cells with MSK. Individually sorted polyploid cells, obtained after 72-h treatment with 25 nmol/L MSK, followed by 72 h in fresh drug-free medium, seeded in separate wells in a 96-well microtiter plate, and allowed to grow for up to 14 d in drug-free medium. A, survival curve showing the number of clones (seeds) containing living cells during the 14-d lifetime span that followed the seeding of individual polyploid cells. B, representative examples of giant living cells (clones) observed under the microscope at the times indicated (top). Cell morphology corresponds to large polyploid cells, which endoreplicate in absence of cytokinesis. Bar, 100 μm. At equivalent periods, all the cells that were treated continuously with MSK (without withdrawal of the drug after a 72-h continuous treatment) were committed to cell death (see Fig. 4A). suggesting that they were entering mitosis (endoreplication) without cytokinesis. MSK was lethal for most of the cells, whereas a small number, with seemingly increased aneuploidy, survived.

Discussion

We have shown that MSK produced polyploidy in HCT-116 (p53+/+) colon carcinoma cells as these cells were only transiently halted at the cell cycle checkpoints. During a normal cell cycle, polyploidization can be prevented by a p53-dependent G1 checkpoint, the G2 checkpoint, and the mitotic spindle checkpoint (10). Moreover, polyploidy can be avoided by preventing these cells from reentering mitosis by an additional “tetraploidy checkpoint” (11, 35). This “tetraploidy checkpoint” would require, together with an intact spindle checkpoint, the presence of active p53, which is fundamental for postmitotic G1 arrest and the prevention of polyploidy upon aberrant exit from mitosis (11, 13). After the withdrawal of MSK, a decrease in p53 levels was observed in HCT-116 (p53+/+) cells. These results are consistent with previous observations that both p53 accumulation and a prolonged mitotic arrest are required for efficient postmitotic G1 arrest (13). MSK appeared to “facilitate” the reentry of cells into mitosis, thus causing mitotic catastrophe. However, HCT-116 (p53−/−) cells, which are defective in checkpoint controls (17), died from G2-M after treatment with MSK by p53-independent apoptosis (36). This finding is in keeping with the observation that p53 deletion in HCT-116 cells leads to greater apoptosis induced by some, but not all, antitumor drugs (18).

Regardless of the presence of active caspase-3, HCT-116 (p53+/+) cells treated with MSK were chiefly committed to cell death by necrosis rather than apoptosis. Here, we show massive necrosis in cells bearing wild-type p53 and active caspase-3. It has been suggested that, when mitotic catastrophe occurs after DNA damage, cells are most likely to undergo slippage and multinucleation followed by nonapoptotic cell death (35), because apoptosis-prone cells, bearing damaged DNA, die before entering mitosis. Both caspase-dependent and caspase-independent mechanisms of cell death after mitotic catastrophe have been reported (7, 8, 37–39), which may end in either necrosis or apoptosis (3, 5). The mode of cell death would be determined by the cell context. The down-regulation of p53 by MSK and the large cell size, which were the result of damaged cells reentering mitosis together with the absence of cytokinesis, might produce cells that die during the abrupt onset of a general malfunction, including permeabilization of cell membranes. This notion was substantiated by the high uptake of PI in the presence of only 3% staining with Annexin V-Fluos. Our results suggest that the rate of necrosis is higher than that of apoptosis, which induces MSK-treated HCT-116 (p53+/+) cells to undergo necrosis even in the presence of active caspase-3, whereas in p53−/− cells apoptosis was mediated by caspase-2. When treated with MSK, HCT-116 (p53+/+) cells did not exhibit significant ΔΨm dissipation (Fig. 4D), which is in line with the absence of apoptosis in these cells. In contrast, the homologous p53−/− cells showed a slight, although significant, decrease in mitochondrial potential, in accordance with a previous observation that tetraploid cells treated with the DNA-damaging agent cisplatin manifest less pronounced ΔΨm dissipation (11).

When p53 is activated in response to some cell cycle errors (1, 10), proapoptotic pathways can also be activated. In contrast, we have shown that the inhibition of p53 expression by MSK results in the generation of a polyploid state, whereas the enhancement of p21WAF1 expression contributes to this state (Fig. 3). This observation agrees with the finding that p21WAF1 is not only a mediator of p53 tumor suppression (40) but also a negative regulator of p53 stability (41).

Although it has been proposed that tetraploidization ends in apoptosis as a safeguard mechanism of cells (11), we conclude that necrosis could replace this process. This finding is not at variance with tetraploidy causing a shift toward resistance to apoptosis (11). It is worth noting that HCT-116 (p53−/−) cells are not necessarily polyploid (Fig. 1B; ref. 17). Therefore, additional genes are involved in preventing polyploidy (42). Although p53 is usually
activated after DNA damage and it is essential to control cell death (17, 43), our results suggest that direct inhibition of p53 or its target genes by MSK is involved in the mechanism by which cells reenter mitosis. The inhibition of Sp1 binding to the promoters of several p53 target genes, such as the \( p21^{\text{WAF1}} \) gene and certain proapoptotic genes, by MTA abrogates the transcriptional induction of these genes by p53, a mechanism that could account for some of the tumor-suppressing and antiapoptotic effects of this drug (34). Based on our results, we propose that this inhibition also occurs with the structurally related MSK, which shows improved therapeutic characteristics (26). We observed the inhibition of p53 transcription (Fig. 3) and the concomitant reduction of p53 protein levels, together with an increase in cyclin B, in addition to other changes, after treatment of cells with MSK (Fig. 5). These findings indicate that p53 did not cause efficient transcriptional activation of some key genes in MSK-treated p53\(^{+/+}\) cells, and thereby an increase in “the rate of spontaneous apoptosis” (9) did not occur, which we have made evident when MSK-treated cells were incubated in drug-free medium.

The MSK-induced inhibition of p53 transcription in HCT-116 (p53\(^{+/+}\) cells was accompanied by unremitting levels of activated \( p21^{\text{WAF1}} \) when cells were allowed to grow in drug-free medium, with a corresponding reduction in the proteins levels, which were not sufficient to prevent the overtaking of the G1 and G2-M checkpoints, with some cells reentering faulty mitosis. These observations are consistent with previous reports that neither \( p21^{\text{WAF1}} \) nor 14-3-3\( \) are sufficient to prevent mitotic catastrophe (14). Our results are also in line with the report that exposure of transformed cells to DNA-binding drugs results in an induction of \( p21^{\text{WAF1}} \) levels and increased ploidy (44). What is peculiar about the effects of MSK is that p53 protein levels were sufficiently reduced to drive cells to act as if they were deficient in wild-type p53, although our results indicate that their behavior is not equivalent to the effects of MSK observed in HCT-116 (p53\(^{-/-}\) ) cells. MSK induced p53\(^{-/-}\) cells to stop only transiently at the distinct checkpoints and to undergo apoptosis from G2-M. The main reason for this situation, compared with p53\(^{+/+}\) cells, might be the known control of the G1 checkpoint by p53. The differences in the effects of MSK on p53\(^{+/+}\) and p53\(^{-/-}\) cells are consistent with the report that p53 is not required for apoptosis induced by DNA damage (45) and also with that the effects of MSK on transcription (Fig. 3) have to be more complex that the direct reduction in p53 levels.

Although most of the HCT-116 (p53\(^{+/+}\) ) cells eventually died in a time-dependent way, as shown by a clonogenic assay, some were still alive after 14 days in fresh drug-free medium, which, added to the previous 72 h in the presence of the IC\(_{50}\) for MSK plus incubation for 72 h in drug-free medium, represents a lifespan of at least 20 days. The assay results shown in Fig. 6 cannot be considered sensu stricto a clonogenic assay because cells were not dividing (forming a clone) but undergoing several rounds of endoreplication and mitosis in the absence of cytokinesis, with polyploidy frequently preceding aneuploidy (13). It is worth noting that surviving cells might undergo an induced, yet time-dependent, enhancement of \( p21^{\text{WAF1}} \) protein levels, which has been identified as a major cause of genetic instability and tumor progression (44).

Connections between the mitotic spindle assembly, p53 status, arrest of the cell cycle at checkpoints, and aneuploidy have been described (11, 13, 46, 47). However, there are several reports indicating that changes in the transcriptome induced by DNA-binding drugs facilitate the overtaking of these and other checkpoints and thus contribute to an increase in polyploidy and mitotic catastrophe (7, 16, 18, 37, 48, 49). HCT-116 (p53\(^{-/-}\) ) and HCT-116 (p53\(^{-/-}\) ) cells treated with MSK showed distinct responses (necrosis in p53\(^{+/+}\) versus apoptosis in p53\(^{-/-}\) cells, which sometimes underwent secondary necrosis) regardless of the presence of active caspase-3 in the former. Therefore, it follows that the link between mitotic catastrophe and the apoptotic machinery remains partially elusive and requires further characterization. The distinct responses of HCT-116 cells to MSK-induced cytotoxicity suggest that the appearance of apoptosis after DNA damage depends not only on the concentration of the drug, as it has been suggested previously (45, 48), but also on the genetic background of the cell beyond the p53 status.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Marc Bataller, Carmen Méndez, José A. Salas, et al.


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