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Letter to the Editor

Induction of apoptosis in human osteosarcoma Saos-2 cells by the proteasome inhibitor MG132 and the protective effect of pRb

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Dear Editor

Osteosarcoma is the most frequent malignant bone tumor, with a peak manifestation during the second and third decades of life.[1] Negative expression of pRb[2] or p53,[3] because of deletion and/or rearrangement of their relative genes, plays an important role in the pathogenesis of human osteosarcomas. In recent years, a class of new agents, now known as proteasome inhibitors,[4] has aroused great interest for its therapeutical potential in the treatment of cancer and inflammation.[5] The multicatalytic complex 26S proteasome is involved in the degradation of many proteic factors that play a role in cell cycle control, tumor growth and apoptosis.[6] Proteasome inhibitors have been shown to be effective in cancer treatment, an ability which seems to be correlated with efficacy in the induction of apoptosis in tumor cells. We have studied the effect exerted on human osteosarcoma Saos-2 cells by MG132, a membrane-permeable peptide aldehyde, which inhibits the chymotrypsin-like activity of the proteasome and acts as a powerful apoptotic agent in a number of tumor cells.[7–10] This paper provides new insights to clarify the mechanism of its apoptotic effect.

Our results demonstrate that MG132 strongly reduced Saos-2 cell viability in a time- and dose-dependent manner with an initial lag phase of about 16 h (Figure 1a). After 48 h of treatment, cell viability, measured by MTT assay as previously reported,[11] decreased to 50% of control with 0.5 μM MG132 and to only 20% with 1.0 μM. Under light microscopy, nonviable cells had a rounded shape and detached themselves from the substrate. The cytotoxic effect of MG132 was because of the induction of apoptosis, as demonstrated by acridine orange/ethidium bromide staining,[12] which under fluorescence microscopy showed chromatin condensation and production of apoptotic bodies in MG132-treated cells. Moreover, quantification of the apoptotic effect by flow cytometric analysis (Figure 1b) showed that it was in a good correspondence with the loss in cell viability induced by MG132. In other experiments, we showed that lactacystin, a specific inhibitor of 26S proteasome, also reduced cell viability (80% with 10 μM) by stimulating apoptosis, while inhibitors of other proteases (calpastatin, calpeptin, cathepsin inhibitor) were ineffective on Saos-2 cells (not shown). These results led us to the conclusion that the apoptotic effect was really determined by inhibition of proteasome. Our results also showed that treatment with MG132 resulted in a significantly higher level of reactive oxygen species (ROS), as demonstrated by the measurement of intracellular content of ROS performed with a permeant fluorescent probe.[13] The effect was clearly observed between 4 and 12 h of treatment (Figure 1c). Thus, the increase in the level of ROS anticipated the onset of apoptosis triggered by MG132. Interestingly, it has been shown that ROS induce oxidation of intracellular proteins, and that proteasome is involved in the removal of oxidized proteins.[14] These findings suggested that treatment with MG132 can lead to an accumulation of these proteins and in particular protein-bound carbonyl groups, which are suitable markers for protein oxidation.[15] In agreement with these considerations we demonstrated, using an ELISA kit (Zentech Technologies), that carbonyl proteins increased from 0.25 ± 0.02 to 0.48 ± 0.05 pmol/μg of protein after treatment for 48 h with 1.0 μM MG132. Our results also showed that oxidants were responsible for the apoptotic effect exerted by MG132, since the addition of antioxidants, such as GSH, *N*-acetylcysteine (NAC) or catalase prevented the generation of ROS (not shown), and at the same time, markedly reduced the cytotoxic effect induced by MG132 (Figure 1d). Moreover, treatment with MG132 caused depolarization of the mitochondrial transmembrane potential ($\Delta\psi_m$) (Figure 1e) with the consequent release of cytochrome *c* from mitochondria to cytosol (Figure 1f). It is well known that efflux of cytochrome *c* in the cytosol causes the activation of caspases,[16] the cysteine proteases required for the execution of the cell death program. By Western blotting it is shown that in Saos-2 cells, MG132 induced fragmentation of procaspase-3 and production of the active form of caspase-3 as well as the breakdown of PARP, a specific substrate of caspase-3 (Figure 1f). However, MG132 treatment was unable to induce fragmentation of procaspase-8 (not shown). Caspase-3 plays a crucial role in MG132-induced apoptosis, as suggested by the finding that 100 μM z-VAD, a cell-permeable, broad spectrum inhibitor of caspase activity, protected the cells from the loss of viability induced by MG132 (Figure 1a). At the same time, it suppressed the activation of caspase-3 as well as the consequent degradation of PARP (Figure 1f) and the morphological changes because of apoptosis (not shown). As z-VAD did not prevent the loss of $\Delta\psi_m$ induced by MG132, we suggest that the involvement of mitochondria preceded caspase-3 activation in MG132-induced apoptosis. In addition, our results provide evidence that 10 mM NAC prevented the effects of MG132 on the loss of $\Delta\psi_m$, as well as on the translocation of cytochrome *c* and the activation of caspase-3 (not shown). These findings suggest that all these effects may be a consequence of the increased generation of ROS caused by MG132. Therefore, they can be considered intermediate steps in the MG132-induced pathway leading to apoptosis.

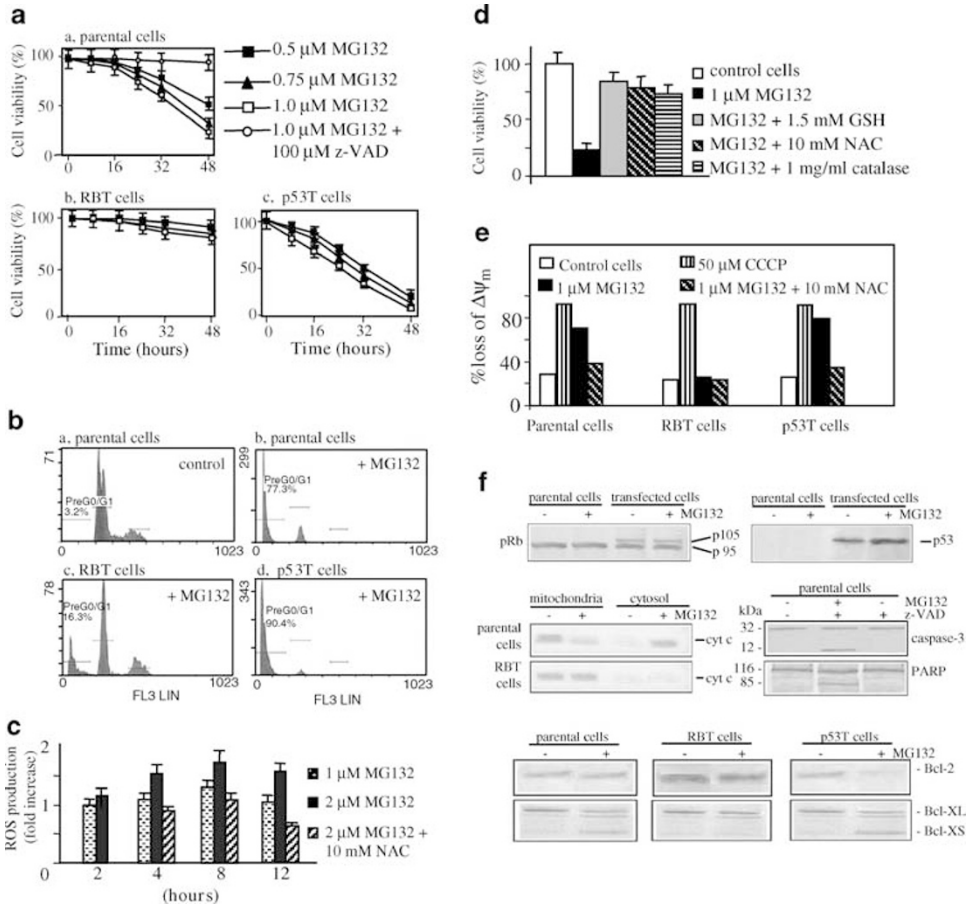

Figure 1 (a) Time course of the effect induced by MG132 on the viability of parental Saos-2 cells (a), in comparison with RB-transfected (RBT) (b), and p53-transfected (p53T) (c) cells. Viability was determined by MTT assay. Saos-2 cells were transfected using FuGene transfection reagent (Boehringer Mannheim). The vectors used were p-CMV-Rb1-puro plasmid and pcDNAhp53-neo. pCMV-puro and pcDNA-neo were used as control vectors. Colonies resistant to puromycin (RBT cells) and to G418 (p53T cells) were cloned and maintained in a medium containing puromycin (0.05 μg/ml) or G418 (200 μg/ml). (**b**) Flow cytometric analysis of Saos-2 cells treated with MG132. Parental, RBT and p53T cells were cultured for 48 h in the absence (a) or presence of 1.0 μM MG132 (b, c and d). Then, DNA was stained with 50 μg/ml propidium iodide. Analysis of stained nuclei was performed using a Beckman Coulter Epics XL cytometer. Data were analyzed with Expo32 software. The results shown in (b) are representative of three independent experiments. The profiles of untreated RBT and p53T cells were similar to that reported for parental cells in control conditions (**a**). (**c**) The effect of MG132 on the generation of radical oxygen species in Saos-2 cells. ROS were quantified using a cell permeant fluorescent probe DCFH-DA (Molecular Probes). DCFH-DA is hydrolized by intracellular esterases to nonfluorescent DCFH, which is then rapidly oxidized by ROS to fluorescent DCF. Parental cells were treated for various times in the absence or presence of MG132 or MG132 plus NAC. At the end, the cells were incubated with 5 μM DCFH-DA for 30 min at 37°C. The dye was then removed and the cells were washed twice with PBS, trypsinized, resuspended in 1 ml of PBS and analyzed using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 525 nm, respectively. Data were analyzed with Expo32 software and reported in the figure as fold of increase with respect to the control cells. (d) The influence of GSH, NAC and catalase on the effect exerted by MG132 on viability of Saos-2 cells. Cells were cultured for 48 h in the presence of 1.0 μM MG132. GSH, NAC or catalase were added as indicated in the figure. (e) The effect of MG132 on mitochondrial transmembrane potential ($\Delta\psi_m$). Quantification of $\Delta\psi_m$ was performed using 3,3-dihexyloxacarbocyanine iodide (DiOC6, Molecular Probes, Eugene, OR, USA), a fluorochrome which accumulates in the mitochondrial matrix under the influence of $\Delta\psi_m$. In some experiments, tetramethylrhodamine ethyl ester (TMRE) was employed as a probe. The results were similar to those found using DiOC6. Parental, RBT or p53T cells were treated for 48 h with MG132 in the absence or presence of 10 mM NAC. The cells were then incubated with 40 nM DiOC6 for 20 min at 37°C, washed twice with PBS, and analyzed in a Beckman Coulter Epics XL flow cytometer with excitation and emission settings of 488 and 525 nm, respectively. The percentage of cells showing lower fluorescence, reflecting loss of mitochondrial transmembrane potential, was determined using Expo32 software. Carbonyl cyanide *m*-chlorophenylhydrozone (CCCP), a protonophore that completely de-energizes mitochondria by dissipating the transmembrane potential, was used as a positive control for maximum $\Delta\psi_m$ disruption. Data are expressed as percentage of cells exhibiting low $\Delta\psi_m$. (f) Expression of pRb, p53, cytochrome *c*, caspase-3, PARP, Bcl-2, Bcl-X_L and Bcl-X_S in parental and transfected Saos-2 cells. Cells were treated for 48 h with 1.0 μM MG132. z-VAD (100 μM) was added as shown in the figure. Protein extracts (about 50 μg) were prepared from parental or transfected Saos-2 cells and analyzed by Western blotting. Proteins were resolved in 7.5% SDS-PAGE to analyze pRb or in 10% in the other cases, then blotted and detected using specific antibodies. Equal loading of proteins was checked by red S Ponceau staining (data not shown)

The proteins of the Bcl-2 family are essential factors in the control of apoptosis.[17] In Saos-2 cells, treatment with MG132 provoked only a modest decrease in the levels of the two antiapoptotic factors Bcl-2 and Bcl-X_L. Apart from Bcl-X_L (31 kDa), Bcl-X gene has been shown to generate another isoform, called Bcl-X_S (21 kDa),[18] which can induce translocation of cytochrome *c* and the consequent activation of caspase-3. As shown in Figure 1f, Bcl-X_S was not visible in untreated Saos-2 cells, but appeared after treatment with MG132. It is possible that these changes in the expression of Bcl-X isoforms contributed to the release of cytochrome *c* from mitochondria. On the other hand, no variations of Bax

were observed in Saos-2 cells under treatment with MG132 (not shown).

Human osteosarcoma Saos-2 cells lack p53 and contain a nonfunctional form of pRb (p95).[19] p53 and the retinoblastoma protein (pRb) are products of tumor-suppressor genes, which are fundamental in the control of cell proliferation. During cell cycle, pRb behaves as a transcriptional repressor by sequestering members of the E2F family, required for the progression of cells from G1 into S phase.[20] pRb is also involved in the terminal differentiation of many cells behaving as a transcriptional coactivator by binding to and potentiating the activity of tissue-specific transcription factors.[21] Finally, pRb has been reported to exert a protective function against apoptosis in differentiating cells, but the mechanism of its action is not clearly defined.[22] Differently, p53 acts as a transcription factor and induces apoptotic activity by regulating a set of genes.[23] To test the effect exerted by pRb or p53 expression on the apoptosis induced by proteasome inhibitors, we obtained stable clones of RB- or p53-reconstituted cells by introducing cDNA vectors endowed with the wild type forms of RB or p53 genes (Figure 1f). Compared to parental cells, all the sublines expressing pRb showed a slower rate of cell growth. Under light microscopy, most of the cells appeared enlarged and flattened, with many dendritic-like protrusions resembling differentiative features. The introduction of RB gene into Saos-2 cells prevented the cells from the effects of 1.0 μM MG132, such as the increase in ROS, the dissipation of $\Delta\psi_m$ and the activation of caspase-3 (not shown). As a consequence, treatment of RB-transfected (RBT) cells with 1.0 μM MG132 determined only a modest cytotoxic effect, which appeared after a prolonged lag phase (Figure 1a). An interesting observation was that the intensity of the band corresponding to Bcl-2 was much higher in RBT cells than in parental cells (Figure 1f). However, since Bcl-2 has been reported to counteract the production of ROS and protect the cells from damages caused by oxidative stress,[24] the increase in Bcl-2 level might be responsible for the reduced cytotoxic effect of MG132 in RBT cells. Clones of p53-reconstituted (p53T) cells showed a susceptibility to MG132-induced apoptosis, which was higher than that shown by parental cells (Figure 1a). This fact was most likely correlated either with an increase in p53 level resulting from the inhibition of proteasome by MG132 or with the decrease in Bcl-2 and Bcl-X_L levels, which was higher in p53T cells than in parental cells (Figure 1f).

Our results demonstrate that the introduction of RB gene into Saos-2 cells exerts a protective influence against apoptosis, while the introduction of p53 potentiates the apoptotic effect induced by MG132. These findings suggest that the efficacy of treating osteosarcomas with proteasome inhibitors may vary in relation to the alterations in RB or p53 genes presented in tumor cells.

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*M Lauricella, A D'Anneo, M Giuliano, G Calvaruso, S Emanuele, R Vento and G Tesoriere**

Department of Cell and Developmental Biology, Section of Biochemistry, University of Palermo, Policlinico, Palermo, Italy

* Corresponding author: G Tesoriere, Department of Cell and Developmental Biology, Section of Biochemistry, University of Palermo, Policlinico, Via del Vespro 129, 90127 Palermo, Italy; E-mail: gtesor@unipa.it

1. Tebbi CK and Gaeta J (1988) Pediatr. Ann. 17: 285 – 300.
2. Benassi MS *et al.* (1999) Int. J. Cancer 84: 489 – 493.
3. Miller CW *et al.* (1990) Cancer Res. 50: 7950 – 7954.
4. Adams J *et al.* (1999) Cancer Res. 59: 2615 – 2622.
5. Goldberg AL, Rock K (2002) Nat. Med. 8: 338 – 340.
6. Ciechanover A (1998) EMBO J. 17: 7151 – 7160.
7. Giuliano M *et al.* (1999) Cancer Res. 59: 5586 – 5595.
8. MacLaren AP *et al.* (2001) Cell Death Differ. 8: 210 – 218.
9. Tenev T *et al.* (2001) Cell Death Differ. 8: 256 – 264.
10. Emanuele S *et al.* (2002) Int. J. Oncol. 21: 857 – 865.
11. Giuliano M *et al.* (1998) Invest. Ophthalmol. Vis. Sci. 39: 1300 – 1311.
12. Dunlop RA (2002) Free Rad. Biol. Med. 33: 894.
13. Ubezio P and Civoli F (1994) Free Rad. Biol. Med. 16: 509 – 516.
14. Shang F *et al.* (2001) Exp. Eye Res. 73: 229 – 338.
15. Buss H *et al.* (1997) Free Rad. Biol. Med. 23: 361 – 366.
16. Jiang X and Wang X (2000) J. Biol. Chem. 275: 31199 – 31203.
17. Rutledge SE (2002) Curr. Opin. Chem. Biol. 4: 479 – 485.
18. Minn AJ (1996) J. Biol. Chem. 271: 6306 – 6312.
19. Shew JY (1990) Proc. Natl. Acad. Sci. USA. 87: 6 – 10.
20. Harbour JW and Dean DC (2000) Genes Dev. 14: 2393 – 2409.
21. Morris EJ and Dyson NJ (2001) Adv. Cancer Res. 82: 1 – 54.
22. Hickman *et al.* (2002) Curr. Opin. Gen. Dev. 12: 60 – 66.
23. Shen Y and White E (2001) Adv. Cancer Res. 82: 55 – 84.
24. Hockenbery DM *et al.* (1993) Cell 75: 241 – 251.