New small molecules, ISA27 and SM13, inhibit tumour growth inducing mitochondrial effects of p53

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Background: p53 is a transcription factor with tumour suppressor properties, which is able to induce mitochondrial apoptosis independently of its transcriptional activity. We recently synthesised two new compounds (ISA27 and SM13), which block p53-MDM2 interaction and induce apoptosis in p53 wild-type (WT) tumour cells. The aim of this study was to verify the effectiveness of these compounds in tumours carrying a mutated form of p53 gene with no transcriptional activity.

Methods: In vitro we evaluated the effectiveness of our compounds in cancer cell lines carrying WT, mutated and null p53 gene. In vivo study was performed in Balb/c nude mice and the mitochondrial-dependent apoptotic signalling was evaluated by western blot.

Results: Both ISA27 and SM13 reduced cell proliferation and induced apoptosis in vitro in cells carrying either p53 WT or mutated gene, suggesting that its effect is independent from p53 transcriptional activity. On the contrary, SM13 had no effect in a p53 null cell line. In vivo, ISA27 and SM13 induced cancer cell death in a dose-dependent manner through the activation of the mitochondrial-dependent death signalling in p53-mutated cells. In vivo, SM13 reduced tumour growth.

Conclusions: Our study proposes SM13 as anticancer compound to use for the treatment of p53-dependent tumours, even in the absence of p53 transcriptional activity.

p53 is best known as a transcription factor that binds to specific DNA sequences and transactivates a number of genes involved in the regulation of cell cycle arrest and apoptosis (Riley et al., 2008). In addition to this nuclear activity, p53 also possesses transcription-independent biological activities that take place far from the nucleus. Indeed, the overexpression of a mutant p53, lacking most of the DNA-binding domain and completely deficient in transactivation function, can efficiently induce apoptosis in human cells (Haupt et al., 1997; Kakudo et al., 2005). Under a variety of cell-death-inducing conditions, p53 rapidly moves to mitochondria where it induces mitochondrial outer membrane permeabilisation, thereby triggering the release of pro-apoptotic factors from the mitochondrial intermembrane space (Kroemer et al., 2007).

p53 family protein activity is regulated in a complex way, including posttranslational modifications, protein stabilisation, protein–protein interaction and modulation of subcellular localisation (Kim et al., 2002; Harms et al., 2004; Bourdon et al., 2005; Harms and Chen, 2005).

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In particular, p53 binds MDM2, an ubiquitin ligase that ubiquitiniates p53 protein and causes inactivation, nuclear export and degradation of p53 (Moman et al, 1992; Haupt et al, 1997; Geyer et al, 2000). In turn, p53 enhances the expression of MDM2, although MDM2 levels can be also regulated via p53-independent pathways (Phelps et al, 2003). Therefore, MDM2 induction in response to p53 is the major negative feedback loop aimed at blocking p53 proapoptotic function and thus allowing cell survival (Malaguarnera et al, 2007). As most of human cancers are p53-dependent owing to inactivating mutations of p53 gene, over-expression of its inhibitors, reduction of its activators or inactivation of its downstream targets (Green and Kroemer, 2009), the possibility to disrupt the MDM2-p53 interaction to enhance p53-dependent inhibition of cancer cell proliferation and survival, has become an attractive goal for cancer therapy (Brown et al, 2009). In the last years, several potent small molecules, which disrupt MDM2-p53 interaction, have been developed and one of them, Nutlin-3, has entered early phase clinical trials (Shangary and Wang, 2009). In the clinical scenario, different cancers express different levels of p53 and also p53 mutant variants, leading to resistance to p53-targeting compounds. On the basis of these findings, we recently synthesised two new compounds: (3R,7aR)-6-(4-chlorobenzy])-1H-spiro[imidazo[1,5-c]thiazole-3,4'-indoline]-2',5,7(6H,7aH)-trione (9c) (Gomez-Monterrey et al, 2010), named ISA27, and 5-bromo-3'- (cyclhexane carbonyl)-1-methyl-2-oxospiro[indoline-3,2' -thiazolidine] (4m) (Bertamino et al, 2013), named SM13. These compounds are able to induce apoptosis of human cancer cell lines both in vitro and in vivo (Gomez-Monterrey et al, 2010; Bertamino et al, 2013; Costa et al, 2013). SM13, in particular, is a promising anticancer compound, which has proven to be more effective than Nutlin at lower doses in different tumour cell lines (Bertamino et al, 2013). We have demonstrated that SM13 inhibits MDM2-induced p53 degradation and activates apoptotic events in vitro in human tumour cells carrying a wild-type (WT) p53 gene (Bertamino et al, 2013). So far, its efficacy has never been tested in vivo. Furthermore, the efficacy of these compounds has never been tested in tumours bearing p53 variants-lacking transcriptional activity. It is possible to speculate that in these cancers, p53 accumulation can still be effective to inhibit cancer growth by enhancing mitochondrial apoptosis. The present study was therefore undertaken to verify whether SM13 and ISA27 retain effectiveness in cancer cells with mutated p53. Also, as SM13 was never tested in vivo, we sought to verify whether the compound is as efficient in vivo as in vitro.

**MATERIALS AND METHODS**

**Cell culture.** MCF7, which express a WT variant of p53, KAT-4, which bears a p53 variant mutated at codon 273 (CGT→CAT; Arg→His), BHT101, which expresses a p53 mutant at codon 251 (ATC→ACC; Ile→Thr) and FRO cells, which do not express p53 at all, were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C in 95% air – 5%CO2.

**Compounds.** Lyophilised ISA27 and SM13 were dissolved in absolute DMSO and used for the treatment of control mice.

**Immunoprecipitation and western blot.** Immunoprecipitation and western blot analysis were performed as described previously (Iaccarino et al, 2006; Sorrentino et al, 2008, 2009). Anti-p53, MDM2, Bax, Cytochrome c, Caspase 9 and Actin antibodies were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany); anti-cleaved caspase 3 antibody was from Cell Signalling (Danvers, MA, USA).

**Cell proliferation and DNA synthesis assay.** Cell proliferation and DNA synthesis assays were performed as described previously (Santulli et al, 2011).

**Tunel assay.** Apoptosis was evaluated in KAT-4 cells after treatment with SM13, using the DeadEnd Colorimetric Tunel System from Promega (Madison, WI, USA), following the manufacturer’s instructions. Results are expressed as mean ± s.d. of apoptotic nuclei.

**In vivo study design.** Experiments were carried out, in accordance to NIH guidelines for Animal Investigation, in 6-week-old BALB/c nude mice (Charles River Italia, Calco, Italy), which had access to food and water ad libitum. For tumour formation, a suspension containing 2×10^6 KAT-4 cells in 200 µl of DMEM were injected subcutaneously in the dorsal side of nude mice, as previously described (Sorrentino et al, 2009). Animals were anesthetised using isofluorane 2%. We used mice that developed tumours of ~6mm in diameter by 2 weeks. Mice were divided into four groups (5 mice per group) and administered twice a week for 2 weeks with intra-tumour or intraperitoneal injections (IP) of the specific treatment (ISA27 and SM13).

In particular, two groups received intra-tumour injection of ISA27 or SM13 either at low (low: 1 mg kg⁻¹) or high dosages (high: 3 mg kg⁻¹); another group received IP of 5 mg kg⁻¹ of the compounds and; the control group received intra-tumour or IP of DMSO. The IP of DMSO did not modify tumour growth with respect to intra-tumour injection, thus in figures we include intra-tumour injection of DMSO as control for treated tumours. Tumour growth was measured by caliper twice a week and expressed as tumour volumes in mm³ according to the formula ‘Volume = (width)^2 x length/2’. At the end of the treatment, mice were killed by cervical dislocation and tumours were processed for biochemical or histological analysis. The Federico II University Ethical Committee for Animal Studies approved all in vivo experimental protocols.

**Real-time PCR.** Total RNA from tumours was isolated using Trizol reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) and cDNA was synthesised by means of Thermo-Script real-time polymerase chain reaction (RT-PCR) System (Invitrogen, Life Technologies), following the manufacturer’s instruction. After transcription reaction, RT quantitative PCR was performed with the SYBR Green RT PCR master mix kit (Applied Biosystems, Life Technologies), as described previously (Ciccarelli et al, 2011). Primers for cytokines gene analysis were as follows: TNFα: forward, 5'-CCAGGAGAAGGCTGACCTCCT-3' and reverse 5'-CGATAAAGGGTGTCAGATAA-3'; VEGF: forward, 5'-CCAGCGTGCTGATACAGATAGA-3' and reverse 5'-TTTCTTGCGTTCTTTTCTTTTT-3'; MMP-9: forward, 5'-GCTCGTGATCCCACATTACT-3' and reverse 5'-AACACACGGTTGTTGCTTC-3'; IL-1β: forward, 5'-GCTCTGGCCCTCAAGGAAGAAAT-3' and reverse 5'-GGAAAGACACAGTTCCTGAGTGAG-3'; P33: forward, 5'-TGAACCCGGGGCCATCCTC-3' and reverse, 5'-GGCAACAAAGCAGACACCTA-3'; p21: forward, 5'-GCGGTCAGAAGGAGGATG-3' and reverse, 5'-GGACGGCAAAAGCAGATTTA-3'.

All values obtained were normalised to the values obtained with the 18S primers. The results are expressed as the relative integrated intensity.

**Histology and Immunocytochemistry.** Paraffin-embedded sections were processed for the triple-layered immunocytochemical peroxidase antiperoxidase method (Sorrentino et al, 2012). PCNA (Sigma Aldrich, Milan, Italy) and Cleaved caspase 3 (Abcam, Cambridge, UK) antisera were used to analyze cell proliferation and death, respectively. The peroxidase was revealed in presence of 0.03% hydrogen peroxide and of an electron donor, 2.5%
diaminobenzidine, which becomes visible as a brown precipitate. For negative controls, the primary antiserum was omitted. Sections were then viewed with an Eclipse E1000 Fluorescence Microscope (Nikon, Milan, Italy) and acquired using Sigma Scan Pro software (Jandel), as described previously (Cittadini et al., 2009). For toxicity assay, Masson trichrome staining was performed in paraffinized sections from the liver, kidney and lung of mice treated with IP of ISA27 or SM13 and control mice, as previously described (Santulli et al., 2012).

Statistical analysis. All values are presented as mean ± s.e.m. Two-way ANOVA was performed to compare the different parameters among the different groups. A significance level of P < 0.05 was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism Software (San Diego, CA, USA).

RESULTS

Effects of ISA27 in different tumour cell lines. We evaluated the effect of ISA27 on p53 levels and activation of caspase 3 in MCF-7 (carrying p53 WT gene), BHT-101 and KAT-4 cells (carrying p53 mutated gene). In all these cell lines, ISA27 increases both p53 and cleaved caspase 3 levels (Figure 1A), suggesting that it inhibits MDM2-dependent p53 degradation and increases apoptotic events in all cell lines. As ISA27 exerts the same effect in p53 WT (MCF-7) and p53-mutated cells (KAT-4 and BHT-101) and given the hotspot mutation of p53 within the DNA-binding domain in KAT-4 and BHT-101 cells, which inhibits its transcriptional activity (Blagosklonny et al., 1998), the effect of ISA27 in the regulation of apoptosis should be independent from p53 transcriptional activity. We therefore performed the study in KAT-4 cells, which represent a well-established model for cancer studies in our laboratory (Sorriento et al., 2009). We first confirmed the effectiveness of ISA27 to disrupt MDM2/p53 binding in KAT-4 cells by co-immunoprecipitation assay. Supplementary Figure S1A shows that MDM2-precipitated p53 and ISA27 reduced this phenomenon. ISA27-dependent increase of p53 levels is due to the inhibition of protein degradation rather than the induction of p53 gene expression. Indeed, p53 gene expression levels, evaluated by RT PCR, were unchanged in treated cells compared with controls (Supplementary Figure S1B). To evaluate the effect of ISA27 on tumour cell growth, we studied in vitro cell proliferation indicators such as cell number and DNA synthesis. ISA27 inhibits both cell proliferation (Figure 1B) and DNA synthesis (Figure 1C) in a time-dependent manner. This finding suggests that ISA27 is able to induce p53-dependent apoptosis also in KAT-4 cells harboring the p53 variant.

Effects of ISA27 on KAT-4 cell growth in vivo. To confirm in vitro data, we tested the effect of ISA27 in vivo. In BALB/c nude mice, the injection of 2 × 106 KAT-4 cells in the dorsal lateral region results in the development of a ∼ 6-mm diameter tumour in 2 weeks, in about 70% of mice. Tumours were treated with different doses of ISA27 by means of IP or intra-tumour injection, as described in Materials and Methods. The effect of ISA27 on tumour growth appears to be dose-dependent. Indeed, intra-tumour injection of high doses (3 mg kg−1) leads to a progressive regression of tumour, whereas low doses (1 mg kg−1) or intraperitoneal treatment (5 mg kg−1) can only delay tumour growth (Figure 2A). At the end of the treatment, mice were killed and tumours were taken for biochemical and histological analysis. We first evaluated p53 and MDM2 levels by western blot to confirm the effectiveness of the treatment in vivo. The p53 levels were increased in all treated tumours (Figure 2B). Also, MDM2 levels were increased in treated tumours with respect to control (Figure 2B), probably as a compensatory response to the increased levels of p53. We then evaluated apoptosis and proliferation in tumours by western blot. Figure 2B shows that ISA27 increased cleaved caspase 3 levels and reduced the phosphorylation of RB in a dose-dependent manner. These results were confirmed by immunohistochemical analysis in paraffin-embedded sections (Figure 3A and B). ISA27-treated tumours show dose-dependent increase of cleaved caspase 3 levels (Figure 3A) and a reduction of PCNA expression, marker of cell proliferation (Figure 3B). These data confirm that ISA27 induces apoptosis also in vivo in a dose-dependent manner.

Effects of SM13 on KAT-4 cell proliferation in vitro. The effectiveness of the nutlin-derivate compound, SM13, in the regulation of apoptotic signalling in a p53 WT tumour cell line (MCF-7) has already been demonstrated. Here we evaluated its effects also in a p53 mutant cell line, KAT-4. We first confirmed the effectiveness of SM13 to disrupt MDM2/p53 binding by co-immunoprecipitation assay. Supplementary Figure S2A shows that MDM2 precipitated p53 and SM13 reduced this phenomenon. We then tested the effect of SM13 on tumour cell growth in vitro. SM13 reduced KAT-4 cell proliferation both at 24 and 48 h from starting treatment (Figure 4A). Giving the mutation of p53 gene in KAT-4 cells, which inhibits its transcriptional activity, we...
evaluated the effect of SM13 on mitochondrial-dependent apoptotic signalling. Indeed, recent studies have shown that interactions of p53 with various members of the Bcl-2 family cause mitochondrial-mediated apoptosis in a transcription-independent manner (Speidel, 2010; Ha et al., 2013). Accordingly, our data showed that SM13 induced p53 levels, increased the expression of the proapoptotic protein Bax, leading to the release of cytochrome c (Figure 4B). This event leads to the activation of both Caspase 9 and Caspase 3, thus, inducing cell death (Figure 4B). Accordingly, also the levels of Cyt-c in cytosolic extracts were increased (Supplementary Figure S2B). We have showed above that ISA27 induced an increase of p53 levels by inhibiting protein degradation without modifying its gene expression levels. We demonstrated that also SM13 did not modify gene expression of p53 (Supplementary Figure S2C), thus confirming that SM13 rather inhibits p53 degradation. To confirm the apoptotic effect of SM13, we evaluated DNA fragmentation through a Tunel assay. Images show that treatment with SM13 induced apoptotic events in tumour cells (Figure 4C). All together, these data suggest that SM13 is able to induce apoptosis in tumour cells by activating mitochondrial apoptotic signalling. To assess the specificity of SM13 effectiveness in the regulation of p53-dependent apoptosis, we evaluated its effects in FRO cells, a tumour cell line which does not express p53 (Wolf and Rotter, 1985; Namba et al., 1995). Figure 4D shows that SM13 increased cleaved caspase 3 levels in KAT-4, but not in FRO cells; thus, suggesting that the ability of SM13 to induce apoptosis strictly depends on p53. Moreover, to confirm the p53 transcription independent effect of SM13, we evaluated gene expression of p53 target genes, p21 and Gadd45. In a p53 WT tumour cell line, MCF7, the treatment with SM13 induced p21 and Gadd45 gene expression, whereas in p53-mutant cell type, KAT-4, such phenomenon was reduced (Figure 4E). These data suggest that the effects of SM13 are strictly dependent on p53, and given the lack of transcriptional activity of p53 (Figure 4E), they are due to the activation of p53-dependent mitochondrial apoptotic pathway.

**Effects of SM13 on KAT-4 cell growth in vivo.** To confirm in vitro data, we evaluated the effect of SM13 on tumour growth in vivo in nude mice. Tumours were treated with IP and intra-tumour injection of SM13. Data show that SM13 is a potent inhibitor of tumour growth in a dose-dependent manner (Figure 5A). Indeed, the intra-tumour injection of SM13 inhibited tumour growth at low dosages and was more efficient at high dosages. Also, the intra-peritoneal treatment strongly delayed tumour growth (Figure 5A). We compared ISA27 and SM13 effects on tumour growth and we found that at equal doses, SM13 is more
effective than ISA27 to inhibit the progression of tumour (Figure 5B). Indeed, when comparing the effect on tumour growth, the three different treatment regimens consistently showed the advantage of SM13 over ISA27. Indeed, the intra-peritoneal treatment with SM13 (5 mg kg\(^{-1}\)) more efficiently delayed tumour growth compared with ISA27 (\(\text{CTRL} 77 \pm 2.6\% \) vs \(\text{SM13} 56 \pm 2\% \) at 14 days, \(P < 0.05\)). Similarly, the intra-tumour injection at low dosages of SM13 (1 mg kg\(^{-1}\)) is more effective than the treatment with low dosages of ISA27 (SM13: \(\text{CTRL} 85 \pm 2\% \) vs \(\text{SM13} 68 \pm 3.4\% \) at 14 days, \(P < 0.05\)). Finally, the intra-tumour injection of high dosages of SM13 (3 mg kg\(^{-1}\)) induces tumour regression more efficiently than ISA27 (\(\text{CTRL} 98 \pm 1.6\% \) vs \(\text{SM13} 80 \pm 1.6\% \) at 14 days, \(P < 0.05\)).

It was already evidenced that ISA27 and SM13 showed different minimal inhibitory concentration 50%, IC\(_{50}\), (SM13: 0.03 \(\mu\)M \(\pm 0.01\); ISA27: 0.40 \(\mu\)M \(\pm 0.02\)) (Gomez-Monterrey et al., 2010; Bertamino et al., 2013) demonstrating that SM13 is 10-fold more potent than ISA27, and this is in agreement with their different effect on tumour growth at the same doses. Thus, besides the good anticancer properties of ISA27, our data show that SM13 is more efficient compared with ISA27 to inhibit tumour growth in vivo.

To confirm the mechanism by which SM13 induced apoptosis, we evaluated its effect on mitochondrial-dependent apoptotic signalling by western blot. SM13-dependent increase of p53 induces activation of Bax, release of cytochrome c from mitochondria and activation of caspases 9 and caspase 3. Images are the mean of three independent experiments. The ability of SM13 to induce apoptosis was evaluated in FRO cells, a tumour cell line, which do not express p53. p53 and cleaved caspase 3 levels were evaluated by western blot. SM13 was not able to regulate cleaved caspase 3 levels in FRO cells with respect to KAT-4 cells. Results are representative of three independent experiments. To confirm the effect of SM13 on apoptosis, we evaluated DNA fragmentation through a TUNEL assay. Positive nuclei were counted and results were expressed in graph as mean \(\pm\) s.d. SM13 is able to induce apoptotic events in tumour cells. Images are representative of three independent experiments.

Figure 4. Effects of SM13 on tumour cell growth in vitro. (A) KAT-4 cells were treated with SM13 for 24 and 48 h, and cell proliferation was analyzed. SM13 reduced cell proliferation in a time-dependent manner (\(*P<0.05\) vs control 24H; \(**P<0.05\) vs control 48H). Results are representative of five independent experiments and are presented as mean \(\pm\) s.e.m. (B) To evaluate the mechanism of action of SM13, we analyzed its effect on mitochondrial-dependent apoptotic signalling by western blot. SM13-dependent increase of p53 induces activation of Bax, release of cytochrome c from mitochondria and activation of caspases 9 and caspase 3. Images are the mean of three independent experiments. To confirm the effect of SM13 on apoptosis, we evaluated DNA fragmentation through a TUNEL assay. Positive nuclei were counted and results were expressed in graph as mean \(\pm\) s.d. SM13 is able to induce apoptotic events in tumour cells. Images are representative of three independent experiments. (D) The ability of SM13 to induce apoptosis was evaluated in FRO cells, a tumour cell line, which do not express p53. p53 and cleaved caspase 3 levels were evaluated by western blot. SM13 was not able to regulate cleaved caspase 3 levels in FRO cells with respect to KAT-4 cells. Results are representative of three independent experiments. (E) To confirm the p53 transcription-independent effect of SM13, we evaluated gene expression of p53 target genes, p21 and Gadd45. In a p53 WT tumour cell line, MCF7, the treatment with SM13 induced p21 and Gadd45 gene expression, whereas in p53-mutant cell type, KAT-4, such phenomenon was reduced (\(*P<0.05\) vs control). Results are the mean of five independent experiments and are presented as mean \(\pm\) s.e.m.
injection of low and high doses of SM13 significantly increased p53, Bax, Cit-C, active caspase 9 and active caspase 3 levels in a dose-dependent manner (Figure 5B). Cell death and proliferation were also evaluated by immunohistochemistry analysis of cleaved caspase 3 and PCNA levels in tumours collected from control and treated mice. SM13-treated tumours show a dose-dependent increase of cleaved caspase 3 levels, marker of apoptosis (B) and reduction of PCNA, marker of cell proliferation (A). Images are representatives of three independent experiments.

**DISCUSSION**

Our results show for the first time that ISA27 and SM13 are two potent inhibitors of cancer cell proliferation even in cancer cells bearing a p53 mutated-variant. p53 is a known tumour suppressor, which regulates several events, like growth arrest, senescence and
apoptosis, in response to cellular damage (Levine, 1997; Wu and Levine, 1997). In basal conditions, p53 is expressed at low levels in cells owing to its continuous MDM2-dependent degradation (Levine, 1997; Wu and Levine, 1997). Rapid induction of p53 protein levels by various stress types prevents inappropriate propagation of cells carrying damaged DNA (Moll and Petrenko, 2003). p53 exerts its pro-apoptotic effect in a transcription-dependent manner in the nucleus and in a transcription independent manner in mitochondria (Chen et al, 1996; Vousden and Lu, 2002; Mihara et al, 2003). Recently, MDM2 has emerged as the main regulator of p53 by limiting the p53 tumour suppressor function (Momand et al, 1992; Finlay, 1993; Chen et al, 1996). Indeed, small molecules have been developed recently to disrupt MDM2-p53 binding, which have been proposed as anticancer therapeutic drugs (Shangary and Wang, 2009). In this context, we have designed and synthesised a series of small molecules, which are analogues of spirooxindole-pyrrrolidine nucleus and inhibit MDM2-dependent degradation of p53 by preventing p53/MDM2 binding (Gomez-Monterrey et al, 2010). Among them, ISA27 resulted the most potent compound to regulate p53 activity. We have recently demonstrated that ISA27 induces p53-dependent apoptosis in a human tumour cell line carrying a WT p53 gene (Costa et al, 2013). Here we show that such effect is reproducible also in tumour cells carrying a mutated p53 gene, which prevents p53 ability to activate transcription of target genes. On the basis of ISA27 structure, we designed and synthesised a series of new modified compounds, which should be more selective and effective than ISA27. These analogues of ISA27 were prepared to explore new structural requirements at the thiazolidine domain for the antiproliferative activity and p53 modulation. Among them, the 5-bromo-3’-(cyclohexanecarbonyl)-1-methyl-2-oxospiro[indoline-3,2’-thiazolidine]-4’-carboxylate (SM13) emerged as the most potent compound, as it inhibits cell growth in different human tumour cell lines at submicromolar and nanomolar concentrations (Bertamino et al, 2013). Docking studies confirmed the interactions of SM13 with the well-known Trp23 and Phe19 clefts, explaining the reasons for its binding affinity for MDM2. SM13 at low doses is capable of inducing the accumulation of p53 protein, inducing significant apoptotic cell death (Costa et al, 2013). Here we demonstrated that SM13, better than its precursor ISA27, is a potent inhibitor of tumour growth in cells carrying a mutated p53 gene and is already effective at low dosages. It is known that p53 induces apoptotic events through the regulation of gene transcription in the nucleus. Recently, it has been shown that this protein is also able to induce apoptosis in a transcription independent manner (Figure 8A), which is based on the activation of the mitochondrial apoptotic signalling (Arima et al, 2005). Indeed, it directly induces permeabilisation of the outer mitochondrial membrane by forming complexes with antiapoptotic proteins Bcl-xL and Bcl-2, resulting in the release of cytochrome c in the cytosol. Moreover, it has been shown that p53 translocation to mitochondria occurs earlier after TPA stimulation, leading to mitochondrial dysfunction (Zhao et al, 2005). The specific signalling involved in this transcriptional-independent effect of p53 on apoptosis is still unclear. What is known is that the transcriptional blockade of p53 by z-amanitin induces p53 mitochondrial localisation and Bax accumulation, and activation in mitochondria. Accordingly, here we show that SM13 induces apoptosis of KAT-4 cells, which express a mutated p53 protein lacking the transcriptional activity, by targeting p53-dependent mitochondrial signalling (Figure 8B). Indeed, the SM13-dependent increase of p53 levels, in KAT-4 cells is associated with the increase of BAX, release of cytochrome c, increase of cleaved caspase 3 levels and, finally, cell death. The confirmation that such mechanism strictly depends on p53, either WT or transcription activity deficient, derives from the finding that SM13 has no effect on FRO cells, a cell line that does not express p53 (Wolf and Rotter, 1985). It could be a compensatory response in those conditions, like cancer, in which p53 cannot activate gene transcription, but must induce cell death to block a pathological increase of cell growth. In conclusion, here we demonstrate that both ISA27 and SM13 are effective regulators of p53 in KAT-4 cells. Among them, SM13 seems to be the most effective as it leads to a progressive tumour regression at lower doses. In treating
human tumours, a combination of selected drugs with different mechanisms of action and a low toxicity grade is preferred over monotherapy. In this context, SM13 could be a good anticancer drug to be used in combination chemotherapy, as it is selective and does not generate side effects. In this study, we demonstrated that SM13 has strong pro-apoptotic effects by regulating p53 turnover within the cell and it could be used as a prototype small molecule for cancer therapy. In conclusion, we propose SM13 as a feasible anticancer drug, which could be used alone or in combination with common anticancer agents to ameliorate the response to chemotherapy in a wide set of tumour types characterised by elevated levels of MDM2 and low levels of p53.

Figure 8. p53 transcription-dependent and -independent activation of apoptotic pathway. (A) p53 is activated in response to DNA damage and induces the apoptotic pathway through two alternative mechanisms, one dependent on transcriptional activity of p53 and one independent from transcriptional activity of p53. Indeed, activated p53 can move towards nucleus to activate the transcription of pro-apoptotic genes or move towards mitochondria to induce the release of cytochrome c. (B) Under basal conditions, MDM2 binds p53, thus leading to the degradation of the protein. SM13 inhibits MDM2/p53 binding by competing with p53 for binding to MDM2. SM13 therefore leads to the inhibition of MDM2-dependent degradation of p53 and to the increase of p53 levels. p53 binds and inhibits antiapoptotic proteins (BclXL); thus, inducing Bax levels on mitochondrial membrane, which leads to the release of cytochrome c from mitochondria that on turn activates apoptotic events.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest.
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