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Research Article

Potent induction of wild-type p53-dependent transcription in tumour cells by a synthetic inhibitor of cyclin-dependent kinases

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Abstract. Activation of the p53 tumour suppressor protein by distinct forms of stress leads to inhibition of cellular proliferation by inducing cell cycle arrest or apoptosis. The cyclin-dependent kinase inhibitor roscovitine has been shown to induce nuclear accumulation of wild-type p53 in human untransformed and tumour-derived cells. We analyzed the response of different human tumour cell lines to roscovitine treatment with respect to their p53 status. Striking induction of wild-type p53 protein and dramatic enhancement of p53-dependent transcription, coinciding with $p21^{WAF1}$ induction, was observed in wild-type, but not mutant, p53-bearing tumour cells after treatment with roscovitine. The transcriptional activity of p53 was substantially higher in roscovitine-treated cells than in cells irradiated with ultraviolet C or ionizing radiation, even though all these agents induced a similar amount of p53 accumulation. These results highlight the therapeutic potential of roscovitine as an anticancer drug, especially in tumours retaining a functional wild-type p53 pathway.

Key words. Roscovitine; p53; p21; transcriptional activity; tumour cells.

The p53 phosphoprotein plays a key role in tumour suppression and in the cellular response to different types of stress. In the normal cellular environment, p53 protein is expressed at low levels in an inactive form. However, in response to stress from hypoxia, DNA damage, changes in redox potential, nucleotide depletion or activated oncogenes (reviewed in [1]), p53 is stabilized mainly through extensive posttranslational modifications, including phosphorylation and acetylation [2–4]. Activation of p53 by distinct forms of stress leads to growth arrest, allowing sufficient time for DNA repair, or apoptosis, which eliminates abnormal cells. p53-mediated G_1 arrest depends on the ability of p53 to transcriptionally activate the cyclin-dependent kinase inhibitor $p21^{WAF1}$ [5–7]. It has also been reported that $p21^{WAF1}$ induction and $p21^{WAF1}$-mediated growth arrest can occur independent of p53 following cellular stress [8]. The tumour suppressive function of p53 protein is abrogated by mutations in the p53 gene or by interaction with certain viral or cellular proteins [9]. Mutation in the p53 gene constitutes the most common type of genetic alteration in human tumours [10].

The cellular mechanisms that regulate the cell cycle involve many protein kinases, where cyclin-dependent kinases (CDKs) play one of the most important roles. CDKs are constantly expressed during the cell cycle, but

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their activity is tightly regulated by complex formation with cyclins. There are also natural inhibitors of CDKs, such as $p16^{INK4}$ (inhibitor of cdk4/cyclin D_1–D_3) [11] and $p21^{WAF1}$ (a general inhibitor of CDKs) [5, 12, 13], whose dysregulation has been linked to the development of cancer. Like p53, CDKs and cyclins are involved in crucial pathways that regulate not only cellular proliferation but also apoptosis. Olomoucine and roscovitine, synthetic purine derivates, act as potent inhibitors of CDK1 and related kinases (CDK2, CDK5, erk1 and erk2) by competing for the ATP binding site of the kinases [14–17]. Recent studies have confirmed that olomoucine and roscovitine display inhibitory effects on cell proliferation in a wide variety of cancer cell lines [18–20], predominantly due to inhibition of CDK/cyclin complexes resulting in cell cycle block at G_1/S and G_2/M transitions in a dose-dependent manner. Most cancer cell lines display typical apoptotic features after treatment with these compounds. The inhibitory effect of olomoucine and roscovitine is not restricted to tumour cells. These compounds also arrest normal cells [21] and are able to inhibit viral replication, probably due to CDK inhibition, because some cell cycle proteins are believed to be essential for expression of specific viral genes [22, 23].

The human p53 protein has been shown to be a good in vitro substrate for CDKs. P53 can be phosphorylated at Ser^{315} by CDK2/cyclinA or CKD1/cyclinB, resulting in an enhancement of its sequence-specific DNA binding and transcriptional activity [24, 25]. Surprisingly, recently published results [26] as well as our own unpublished data have shown that treatment of cells bearing wild-type (wt) p53 with CDK inhibitors olomoucine or roscovitine leads to strong nuclear accumulation of endogenous p53 protein. These observations have raised the question of whether the induced p53 protein is capable of transactivating target genes and whether the CDK inhibitor treatment also influences mutant (mut) p53 protein levels.

We report here that the effect of roscovitine on p53 protein accumulation is restricted to cells expressing wt p53. Accumulated wt p53 protein is transcriptionally active and responsible for elevated $p21^{WAF1}$ expression. Interestingly, in comparison to ultraviolet C (UV-C) and ionizing radiation, inducing a comparable amount of p53 protein, roscovitine-treated cells exhibited substantially higher p53 transcriptional activity. Our results support the idea that the phosphorylation of p53 by CDKs could have an inhibitory or a stimulatory effect on wt p53-dependent transcription, depending on cellular context [27, 28].

Materials and methods

Cell culture and treatment

Cell lines established from human breast carcinoma (MCF-7, BT549), human osteosarcoma (HOS), murine fibroblasts (T22lacZ) (kindly provided by Dr X. Lu and Prof. D. P. Lane) and human melanoma (Arn8) (kindly provided by Dr J. Bladyes) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum. For UV-C irradiation, cells were first washed with phosphate-buffered saline (PBS) and then irradiated (40 J/m^2) in the absence of medium using a model 1800 Stratalinker (Stratagene, La Jolla, CA, USA). For ionizing irradiation cells were irradiated (4 Gy, 6 MeV) using a medical linear accelerator Clinac 600C (Varian Medical Systems, Palo Alto, CA, USA). Roscovitine was added from a 50 mM stock solution in dimethyl sulphoxide (DMSO) into culture medium to a final concentration of 20 μM. Control cells received an equivalent volume of DMSO.

Analysis of p53-dependent transcriptional activity

As a measure of p53-dependent transcriptional activity, β-galactosidase activity was determined for the human melanoma cell line Arn8 and the murine fibroblast cell line T22lacZ (both stably transfected with a p53-responsive reporter construct pRGCΔfoslacZ [29]). Cells were lysed by three freeze-thaw cycles in 0.25 M Tris pH 7.5, and lysates were assayed as described in [30].

Antibodies

DO-1, DO-2 and 1801 monoclonal antibodies recognize the N-terminal region of p53 protein [31, 32]; monoclonal antibodies DO-11 and DO-12 recognize different epitopes in the core domain of p53 protein [33]; monoclonal antibodies Bp53-10 and Pab421 recognize the C-terminal region of p53 protein [34–36]. Monoclonal antibody 118 recognizes $p21^{WAF1}$ protein [37].

Polyacrylamide gel electrophoresis and immunoblotting

For direct immunoblotting, total cellular protein lysates were prepared by harvesting cells in hot Laemmli electrophoresis sample buffer. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gel and transferred onto a nitrocellulose membrane in a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell for 2 h at 4 °C applying 150 mA in transfer buffer (240 mM Tris, 190 mM glycine and 20% methanol). Prestained molecular weight markers (Bio-Rad, Hercules, CA, USA) were run in parallel. The blotted membranes were blocked in 5% milk and 0.1% Tween 20 in PBS for 2 h and probed overnight with monoclonal antibodies. After washing three times in PBS plus 0.1% Tween 20, peroxidase conjugated rabbit anti-mouse immunoglobulin antiserum (DAKO, Copenhagen, Denmark) diluted 1 : 1000 was used as the secondary antibody. To visualize peroxidase activity, ECL reagents from Amersham-Pharmacia (Little Chalfont, England) were used according to the manufacturer's instructions.

Transfection experiments

The MCF7-DDp53 cell line was derived from MCF-7 parental cells by stable transfection with plasmid pCMV-neoDDp53 coding for a dominant-negative truncated mouse p53 protein including amino acid residues 1–14 and 302–390 under the control of the CMV (cytomegalovirus) promoter [38] (kindly provided by Prof. D. P. Lane). The control cell line MCF-7neo was derived by transfecting MCF-7 cells with pCMVneo vector. Transfections were performed using the Effectene transfection reagent (QIAGEN, Hilden, Germany) as recommended by the supplier. Stable transfectants were selected at 2 mg/ml G418 sulphate (Gibco BRL, Paisley, Scotland). The expression of DDp53 miniprotein in the MCF-7DDp53 cell line was examined by immunoblotting with Bp53-10 monoclonal antibody. Independently isolated MCF-7DDp53 clones 9, 12 and 14 expressing high levels of DDp53 miniprotein and MCF-7-neo clones 3, 4 and 7 were used.

Results

Roscovitine induces wild-type, but not mutant, p53 protein

First, to determine an appropriate concentration of roscovitine, MCF-7 (wt p53) cells were treated for 12 h with increasing concentrations of roscovitine ranging from 1 to 100 μM and analyzed for p53 protein expression using monoclonal antibody DO-1 (fig. 1A). The concentrations of roscovitine from 20–100 μM were found to affect the level of p53 protein in these cells. As shown at figure 1A, the level of protein expression induced by 20 μM roscovitine was not substantially different from expression induced by 100 μM roscovitine, so that 20 μM concentration was selected for further experiments. Second, the time-dependent increase in p53 expression after 20 μM roscovitine treatment was examined. Incubation periods of 6, 12 and 24 h were chosen, since by 6 h the level of protein expression had reached a steady state (fig. 1B).

We analyzed the expression of p53 protein in MCF-7 (wt p53), BT549 (mut p53) and in HOS cells (mut p53). Although treatment of MCF-7 cells for 6, 12 and 24 h with 20 μM roscovitine resulted in significant accumulation of wt p53 (fig. 2, 1B), no induction of p53 was observed following exposure of p53 mutant cell lines to 20 μM roscovitine at any time point (fig. 2). No correlation was observed between the status of p53 and the viability of cells treated with roscovitine.

Roscovitine induces more transcriptionally active wt p53 than UV-C and ionizing irradiation

The effect of roscovitine on activation of p53 protein was also analyzed in the human melanoma cell line Arn8 and in the murine fibroblast cell line T22LacZ expressing

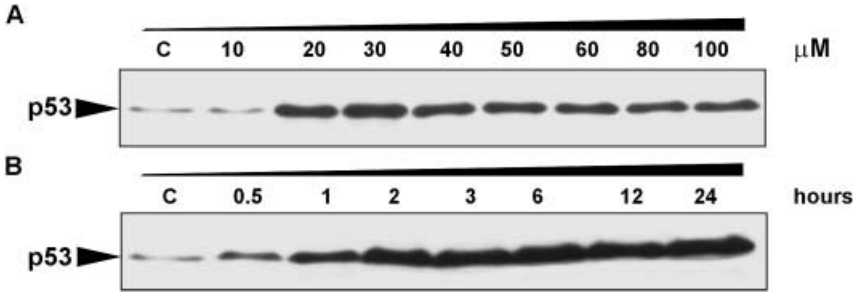

Figure 1. (*A*) Concentration-dependent induction of p53 protein in MCF-7 cells 12 h after roscovitine treatment. An increase in the concentration of roscovitine above 20 μM fails to induce a higher level of p53 protein. (*B*) Time-dependent induction of p53 protein in MCF-7 cells. p53 induction was visible 30 min after roscovitine treatment, reaching a maximum level at 2 h post-treatment. The level of p53 remained constant following longer time periods. Total cell lysates were separated on a 10% SDS gel. Immunoblotting was performed with monoclonal anti-p53 antibody DO-1.

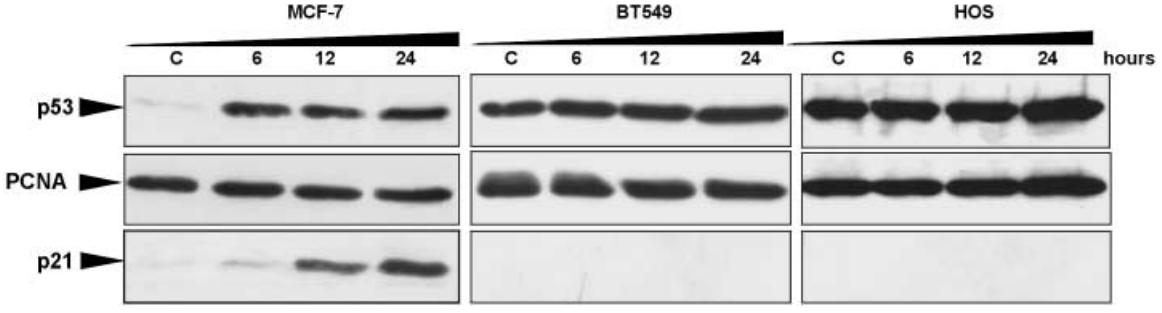

Figure 2. Effect of roscovitine on p53 and $p21^{WAF1}$ induction in cells expressing either wild-type or mutant p53 protein. Total cell lysates were separated on a 12.5% SDS gel. Immunoblotting was performed with monoclonal anti-p53 antibody DO-1, anti-p21 antibody 118 and subsequently with anti-PCNA antibody PC-10, to confirm equal protein loading.

β-galactosidase under the control of a p53-responsive promoter. Induction of wt p53 in these cells treated with 20 μM roscovitine (6, 12 and 24 h) led to activation of the responsive promoter and consequently to expression of β-galactosidase. Roscovitine-treated Arn8 and T22lacZ cells were fixed and examined microscopically for β-galactosidase activity using X-gal substrate [39], resulting in about 35% blue-coloured cells compared with less than 1% of blue-coloured cells in DMSO-treated control cells (data not shown). Total β-galactosidase activity in Arn8 cells was also assessed using a colorimetric assay. Figure 3A shows strong activity of β-galactosidase at 12 and 24 h after roscovitine treatment, providing evidence of transcriptionally active p53 protein in comparison with control cells treated with DMSO. Interestingly, UV-C (40 J/m^2) and ionizing radiation (4 Gy) treatment, inducing a comparable amount of p53 protein as roscovitine (fig. 3B), led to significantly lower p53-transcriptional activity measured using the p53 consensus-regulated reporter gene assay (fig. 3A).

Roscovitine-induced wt p53 activity is responsible for p21^{WAF1} induction

In MCF-7 and Arn8 cells, roscovitine-induced p53-transcriptional activity also led to p21^{WAF1} induction. Accumulation of p53 protein was apparent 4 h after roscovitine treatment, but an enhanced level of p21^{WAF1} protein was not observed until 12 h after treatment with roscovitine (fig. 2). Only wt p53-expressing MCF-7 cells responded to roscovitine with p21^{WAF1} induction (fig. 2). To confirm that p21^{WAF1} induction is p53-responsive, a series of stably transfected MCF-7 clones expressing high levels of a dominant-negative DDp53 miniprotein was established. This protein, consisting of amino acids 1–14 and 302–390 of the mouse p53 sequence, has been shown to bind to the C-terminus of wt p53 and abrogate p53-dependent transcription [38]. Control MCF-7neo clones were also established by stable transfection of MCF-7 cells with the backbone vector pCMVneo without an insert. Clones expressing a high level of DDp53 as well as control clones were treated with roscovitine and assayed for p21^{WAF1} expression using immunoblotting with the p21^{WAF1} specific monoclonal antibody 118. As a result of DDp53 expression, no p21^{WAF1} induction could be detected in MCF-7DDp53 clones (fig. 4).

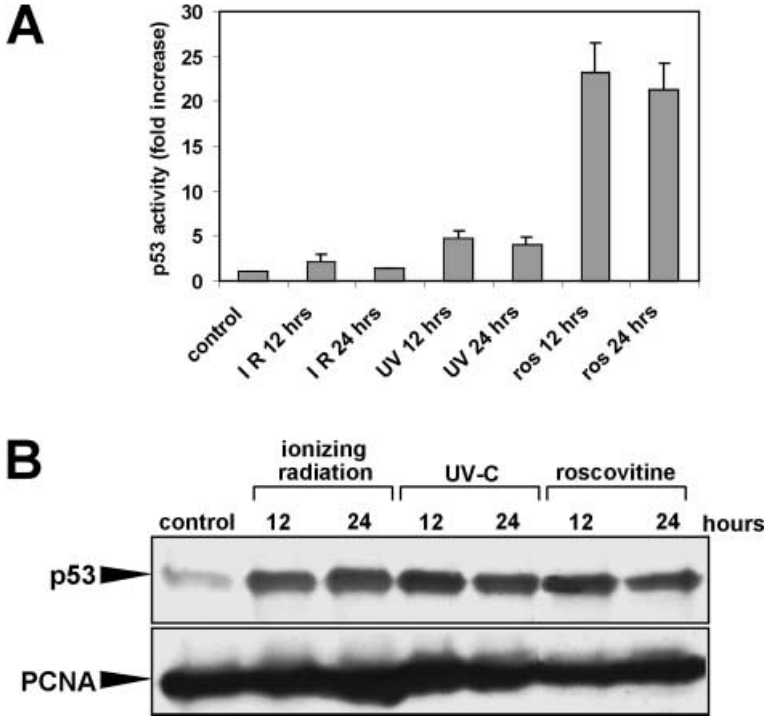

Figure 3. (*A*) Effect of roscovitine and DNA-damaging radiation on p53-dependent transcription of the β-galactosidase reporter gene. Arn8 human melanoma cells were treated with 20 μM roscovitine (ros), 40 J/m^2 of UV-C and 4 Gy of ionizing radiation (IR). After treatment, cells were pelleted, lysed and examined (12.5 μg of protein) for changes by enzyme assay. Note strong activity at 12 and 24 h after roscovitine treatment in comparison with ionizing and UV-C irradiation. (*B*) p53 protein expression following roscovitine, UV-C and ionizing radiation treatment. Cell lysates used for the p53 activity assay were separated on a 10% SDS gel (10 μg of protein per lane). Immunoblotting was performed with monoclonal anti-p53 antibody DO-1 and subsequently with anti-PCNA antibody PC-10, to confirm equal protein loading.

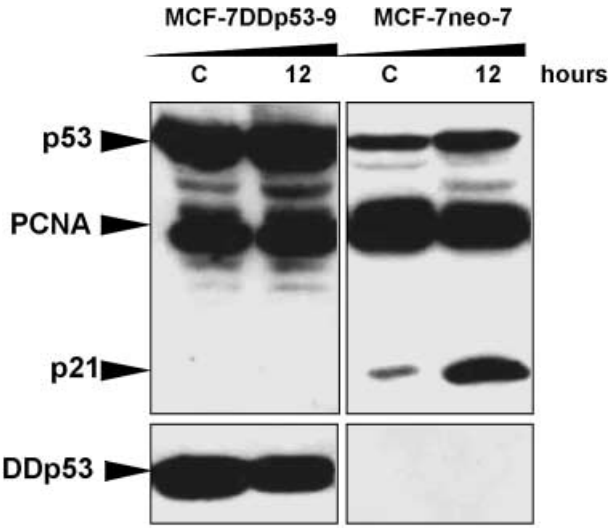

Figure 4. Confirmation of p53-dependent p21^{WAF1} protein induction after 20 µM roscovitine treatment in MCF-7 cells using transfection of dominant-negative truncated mouse p53 protein (DDp53), which disrupts p53 transcriptional activity. Total cell lysates were separated on 12.5% SDS gel. Immunoblotting was performed with monoclonal anti-p53 antibodies DO-1, Bp53-10 (for DDp53 detection), anti-p21 antibody 118 and anti-PCNA antibody PC-10 as a loading control. The left panel shows the MCF-7DDp53-9 clone expressing a high level of DDp53. The right panel shows the MCF-7neo-7 control clone without DDp53.

Discussion

Activation of p53-dependent transcription occurs following exposure of cells to a variety of genotoxic agents. Genotoxic stress can induce accumulation of p53 in the nuclei of mammalian cells and cause either growth arrest or apoptosis in a p53-dependent manner. It has been shown that induction of the p53 pathway can also be activated by nongenotoxic agents, including heat shock or hypoxia. Recently, the cyclin-dependent kinase inhibitor roscovitine has been shown to induce nuclear accumulation of wt p53 in human untransformed and tumour-derived cells [26], although some reports suggest that the level of p53 protein expression is not always in accordance with its transcriptional activity [40–42]. A recent study concluded that roscovitine treatment (at 10 µM concentration) leads only to weak induction of p53 transcriptional activity but synergizes with ionizing radiation in stimulating p53 transcriptional activity, possibly due to interference with a kinase pathway that regulates general RNA polymerase II activity [40].

Our results, using monoclonal antibody DO-1, provide evidence that roscovitine at 20 µM concentration can induce high levels of p53 protein in cells expressing wt p53, but that it does not affect the levels of various mutant p53 proteins. Comparative analysis using a series of monoclonal antibodies (1801, DO-2, DO-11, DO-12, Bp53-10, Pab421) recognizing different epitopes on the human p53 protein (data not shown) demonstrated that the increase in p53 protein level detected by DO-1 and DO-7 (previously used in [26]) monoclonal antibodies, sharing the same phosphorylation-sensitive epitope [43] was not caused by posttranslational modifications of this epitope.

The biochemical activity of p53 largely involves its ability to function as a transcription factor [44]. Arn8 cells, bearing a stably integrated β-galactosidase gene containing a consensus p53 binding site in its promoter, were used to test whether the roscovitine-induced wt p53 exhibits transcriptional activity. An approximately 20-fold increase in p53 activity (fig. 3A) suggests that roscovitine might be sufficient to trigger a regulatory pathway leading to activation of p53-dependent transcription. Interestingly, the total p53 activity induced by 20 µM roscovitine was approximately 4–5 times higher than the p53 activity in UV-C irradiated cells and approximately 8–10 times higher than the p53 activity in ionizing irradiated cells. Immunoblotting analysis revealed a comparable amount of p53 protein induced in Arn8 cells by distinct treatments (fig. 3B). Recently reported studies show that p53 transcriptional activity can be stimulated by phosphorylation at serine 315 by CDK2/cyclinA and that the specific activity of p53 protein can be reduced by inhibiting the CDK pathway with the kinase inhibitor roscovitine in an assay using transiently transfected cells [25]. Conversely, our data obtained in a system with a stably integrated reporter gene (Arn8 cells) show that the roscovitine-induced p53 protein exhibits significantly higher specific transcriptional activity, at least towards certain promoters, compared with p53 protein induced in response to UV-C or ionizing irradiation, suggesting a considerable increase in p53 transcriptional activity even in the presence of a potent CDK inhibitor. This discrepancy might be explained by differences between the p53 activity assays used. Transient transfections may evoke cellular stress which, in turn, activates distinct pathways regulating p53 activity. Moreover, roscovitine exhibits other biological activities, e.g. inhibition of RNA polymerase II-dependent transcription [45], that might influence p53 activity depending on cellular context. For example, roscovitine was found to enhance p53 transcriptional activity in ionizing irradiated cells [40].

Induction of the CDK inhibitor p21^{WAF1}, regulated through p53-dependent and p53-independent mechanisms, is a common feature of growth arrest in different physiological settings. p21^{WAF1} is transiently induced in the course of senescence, damage-induced growth arrest, and terminal differentiation of postmitotic cells. p53 induction by different stress signals is not always associated with p53-dependent transcription of the p21^{WAF1} gene. It has been shown that the anticancer drug suramin increases p53 protein levels without affecting the p53-dependent transcription of the p21^{WAF1} gene [46]. Similar

results were obtained using a potent mammary carcinogen, anti-benzo[g]chrysene-11,12-dihydrodiol-13,14-epoxide in MCF-7 cells. This carcinogen increased the level of p53 protein without induction of $p21^{WAF1}$ protein [47]. Our experiments demonstrate that roscovitine induces a high level of transcriptionally active wt p53 protein that is responsible for induction of $p21^{WAF1}$ protein in MCF-7 cells, as shown in experiments using MCF-7 cells stably transfected with pCMVneoDDp53 vector. As a result of DDp53 expression, the transcriptional activity of p53 and induction of $p21^{WAF1}$ after roscovitine-treatment were abrogated. The dependence of $p21^{WAF1}$ induction on the presence of wt p53 was also confirmed using cell lines expressing different mutant forms of p53 protein treated with roscovitine. None of these cell lines exhibited $p21^{WAF1}$ protein induction.

In some cell systems, the transcriptional activity of p53 protein is required for triggering apoptosis [48]. Roscovitine was revealed to induce apoptosis in the human breast cancer cell line MDA-MB-231 [20], and it has been suggested that roscovitine activates both p53-dependent and p53-independent apoptotic pathways [26]. In our roscovitine-treated tumour cells, apoptosis was also observed using TUNEL and keratin 18 cleavage (data not shown).

Taken together, the data presented here demonstrate that the synthetic CDK inhibitor roscovitine induces endogenous transcriptionally active wt p53, leading to $p21^{WAF1}$ induction. Furthermore, roscovitine-induced p53 protein exhibits higher transcriptional activity compared with p53 induced by DNA-damaging UV-C or ionizing irradiation. These findings highlight the therapeutic potential of roscovitine as an anticancer drug especially in tumours retaining a functional wt p53 pathway, and support the idea that roscovitine could function as a sensitizer of the p53 response to other therapeutic agents [40].

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