**(R)-ROSCOVITINE (CYC202, SELICICLIB) SENSITIZES SH-SY5Y NEUROBLASTOMA
CELLS TO NUTLIN-3 INDUCED APOPTOSIS**

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ABSTRACT

In this study we have analyzed the consequences, on several neuroblastoma cell lines, of combined treatments with (R)-roscovitrine (CYC202, Seliciclib), a CDK inhibitory drug, and nutlin-3, a p53 activating drug. Both compounds were found to synergize, causing significant levels of apoptosis in cultured cells when combined at sub-lethal concentrations. In SH-SY5Y cells, Bcl-XL protein overexpression protected from apoptosis induced by either nutlin-3 alone or the (R)-roscovitrine plus nutlin-3 association, but failed to prevent apoptosis triggered by (R)-roscovitrine alone. Moreover, Western blot studies showed that (R)-roscovitrine increased nutlin-3-mediated p53 stabilization. Therefore, we conclude the role of (R)-roscovitrine in the synergism is basically the sensitization of SH-SY5Y cells to the action of nutlin-3 on p53. The relevancy of this pharmacological synergism envisaging the treatment of neuroblastoma treatment is discussed.

Keywords: (R)-roscovitrine, CYC202, seliciclib, nutlin-3, apoptosis, p53, HDM2, cyclin, cyclin-dependent kinase, cancer

INTRODUCTION

Nutlin-3 is a cis-imidazoline compound that has recently been described as a potent and selective antagonist of MDM2 binding to the p53 protein. Nutlin-3 is thus a promising new molecular scaffold able to unleash the p53 tumor suppressing power in cancer cells [1]. MDM2, HDM2 in human, is encoded by a gene under the positive transcriptional control of p53 [2]. In contrast, HDM2 is a negative regulator of p53 function as it promotes p53 export from the nucleus and its subsequent ubiquitin/proteasome mediated degradation [3]. This negative feed-back loop between the two proteins is targeted by nutlin-3 which, consequently, stabilizes p53.

Cyclin-dependent kinase (CDKs) inhibitory drugs are also being extensively studied as promising agents for cancer therapy [4, 5]. Among the first inhibitors, (R)-roscovitine (CYC202, Seliciclib) has reached phase II clinical trials against breast cancer, various B-cell malignancies and non small cell lung cancer [6-8]. Roscovitine has proven to be an effective inducer of apoptosis in a large diversity of human cells including those from breast cancers [9], chronic lymphocytic leukemia [10] or neuroblastoma [11].

Roscovitine has been shown to be active in combination with various treatments such as irradiation therapy of breast cancer [12] or farnesyltransferase inhibitors in several cancer cell lines [13]. As a matter of fact, the clinical trials of (R)-Roscovitine are conducted as a combination with standard chemotherapy [4, 7, 14]. These antecedents prompted us to explore whether nutlin-3 and (R)-roscovitine displayed some pharmacological synergism in several cell lines derived from human neuroblastoma.

SH-SY5Y is a cell line derived from an abdominal metastasis of a human neuroblastoma in which we have been studying apoptotic processes induced, for instance, by roscovitine [11]. In many neuroblastoma cells, like SH-SY5Y, p53 protein is present and capable to trigger the canonical p53-mediated transcriptional responses and apoptosis [15]. We found that (R)-roscovitine and nutlin-3 synergize in inducing apoptosis in several cell lines derived from neuroblastoma. In SH-SY5Y cells, Bcl-XL overexpression failed to prevent

apoptosis triggered by (R)-roscovitine but protected these cells from nutlin-3 and nutlin-3 plus (R)-roscovitine synergism to a similar extent. This suggests that the apoptotic pathway triggered by nutlin-3 is the one taking place when nutlin-3 and (R)-roscovitine are combined. We conclude that sublethal concentrations of (R)-roscovitine enhance the apoptotic response of neuroblastoma cells triggered by nutlin-3.

MATERIAL AND METHODS

Chemical reagents

(R)-roscovitine was provided by N. Gray, Novartis Reserch Foundation (San Diego, CA, USA). Nutlin-3 was purchased from Cayman (Ann Arbor, MI, USA). AcDEVDAfc (Acetyl-Asp-Glu-Val-Asp-7-amino-4-[trifluoromethyl] coumarin) was obtained from MP biomedicals (Strasbourg, France). The CellTiter 96® kit, containing the MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxy methoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) reagent, and the Cytotox 96® kit, used to determine lactate dehydrogenase (LDH) release activity, were purchased from Promega (Madison, WI, USA). Unless otherwise stated, all other reagents were from Sigma (St. Louis, MO, USA).

Cell line culture and treatment

SH-SY5Y, IMR-5 and IMR-32 cells were grown in DMEM medium from Cambrex (Emerainville, France) supplemented with 2 mM L-glutamine from Eurobio (Courtaboeuf, France) and a 10% volume of fetal calf serum from Invitrogen (Cergy Pontoise, France). Cell cultures were maintained in a 95% air, 5% CO₂, water saturated atmosphere at 37°C. Culture dishes and other plastic disposable tools were supplied by Corning (Corning, NY, USA). SH-SY5Y cell clones transfected with the pcDNA3/Bcl-XL and the pcDNA3 empty vector were obtained and cultivated as reported previously [11, 16]. (R)-roscovitine and nutlin-3 stock solutions were prepared in DMSO at a 20 mM concentration. From these stock solutions, drugs were adjusted in the culture media at the final concentrations reported in the

text and figure legends. DMSO concentration never surpassed 1%, a non toxic concentration in the culture media.

Determination of cell death, cell viability and effector caspase activity

The MTS method allows to measure cell viability while the LDH release determination provides an estimate of cell death level. Both procedures can be carried out in the culture multiwell plates. They were performed according to providers' instructions and as previously described [11]. Trypan blue is another method to assess cell viability. Healthy cells exclude Trypan blue and viability is determined by light microscope counting. Counts were performed on cultures grown in 24 multiwell plates containing $2-3 \times 10^5$ cells/well. After 48 hr of seeding, cells were treated for another 48 hr, then collected and gently dissociated in culture medium. One volume of a 0.4% Trypan blue solution was added to 4 volumes of the cell suspension and cells were counted with a haemocytometer. Apoptotic nuclear morphology was assessed by means of the bisBenzimide (Hoechst 33342) dye and fluorescence microscopy. Effector caspase activation (DEVDase activity) was quantified by measuring the proteolysis of AcDEVDafc, a fluorogenic substrate. Determination of DEVDase activity on 96 multiwell plates was performed as previously standardized [11, 16].

Protein extraction and Western blotting

Cells extracts for Western blotting analysis were prepared as follows. Cells were first washed with PBS (Phosphate Buffered Saline) and lysed by sonication in a buffer containing 1% SDS, 50 mM Tris-HCl (pH 6.8), 1 mM EDTA and a cocktail of protease inhibitors (10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 100 µM Benzamidine). Following room temperature centrifugation at 12000 g for 15 min, the protein extract (supernatant) was obtained. Protein concentration was determined with the DC Protein Assay from BioRad (Hercules, CA, USA) and equal amounts of protein were subjected to electrophoresis in 8-12% polyacrylamide gels. Proteins were then transferred to 0.45 µm nitrocellulose membranes (Schleicher and

Schuell) and membranes were probed with the following primary antibodies: anti-p53 (Catalog # sc-263) (1:2000) and anti-MDM2 (Catalog # sc-965) (1:200) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-p21^{Cip1} (Catalog # OP64) (1:500) from Calbiochem (San Diego, CA, USA) and anti- α Tubulin (1:2000) (Catalog # T5168) from Sigma. Secondary peroxidase conjugated antibodies (Sigma) were used to reveal the antigens by enhanced chemiluminescence (ECL, Amersham).

RESULTS

(R)-roscovitine and nutlin-3 synergize at inducing apoptosis in neuroblastoma cells

The first step to evaluate a putative synergy between (R)-roscovitine and nutlin-3 was to determine the range of concentrations causing SH-SY5Y cell death for each compound, separately. The MTS reduction assay was used to quantify cell viability. This allowed us to define a lethal (37.5 - 50 μ M) and a sublethal (10 μ M) concentration for (R)-roscovitine, and a lethal (5 - 10 μ M) and almost sublethal (2.5 μ M) concentration for nutlin-3, following 48 hr of treatment (Fig. 1A). We next tested the combined addition of (R)-roscovitine (10 μ M) and nutlin-3 (2.5 μ M). A reduction in cell viability, far greater than that caused by each drug used individually at the sublethal concentration, was observed (Fig. 1A). It is known MTS reduction can be caused by cellular events different from cell death. To circumvent this uncertainty, we have systematically validated the MTS results by using the LDH release method [11]. As shown in Fig. 1B, cell death quantification by the LDH release procedure yielded values consistent with those provided by the MTS assay. In conclusion, a pharmacological synergism existed in SH-SY5Y cells.

We followed a similar approach In IMR-5 and IMR-32, two additional cell lines derived from human neuroblastoma. (R)-roscovitine and nutlin-3 were tested and cell death was found to occur in the same range of concentrations previously determined for SH-SY5Y cells. The pharmacological synergism was also tested and found to exist in both cell lines (Table 1).

The MTS reduction and LDH release procedures are quite appropriate to quantify cell viability and cell death in culture plates. However, they do not provide any information about the ongoing type of cell death. In order to assess the mechanism of cell death induced by the combination of (R)-roscovitine (10 μ M) and nutlin-3 (2.5 μ M), two complementary approaches were used. First, bisBenzimide staining and fluorescence microscopy allowed the unequivocal identification of apoptotic nuclei (Fig. 2). Second, the determination of DEVDase activity, by quantification of the cleavage of the fluorogenic substrate AcDEVDafc, provided an estimation of the effector caspase 3 and 7 activity (Fig. 3). An increase of DEVDase activity is considered as an early biochemical marker of apoptosis. A marked increase in DEVDase activity was observed after 12 hr of exposure to (R)-roscovitine (10 μ M) plus Nutlin-3 (2.5 μ M). In these DEVDase experiments, cell death was confirmed at 48 hr using the Trypan blue staining procedure. The cell death ratios were 5.56 ± 1.25 % for (R)-roscovitine (10 μ M), 9.67 ± 1.58 % for nutlin-3, and 46.35 ± 3.22 % for the combined treatment in SH-SY5Y cells. These values of cell death were consistent with those obtained with the LDH release method (Fig. 1B), thus providing full biological significance to the DEVDase activity experiments. Similar results were observed in IMR-5 and IMR-32 cell lines subjected to the combined treatment (Fig. 3). After 12 hr of exposure to (R)-roscovitine (12.5 μ M) plus Nutlin-3 (2.5 μ M) a synergistic activation of effector caspases was found. In conclusion, not only was the cell death process identified as apoptotic but further support of the pharmacological synergism was provided.

(R)-roscovitine acts by sensitizing SH-SY5Y cells to nutlin-3 pro-apoptotic effects

In addition to the 48 hr end point for the cell death and cell viability determinations (Fig. 1), we had also performed measurements after 24 hr of treatment. Taken together, all these results allowed us to compare the time-course leading to cell death in SH-SY5Y cells exposed to these treatments (Fig. 4). As expected from previous data [11], a lethal concentration of (R)-roscovitine killed most of SH-SY5Y cells at 24 hr. On the contrary, a

lethal concentration of Nutlin-3 required 48 hr to kill more than half of the cell population. This was in agreement with the reported 48 hr optimal delay required to detect Nutlin-3 induced apoptosis in other cell types [1]. Interestingly, the time-course of cell death induction by the combined treatment with (R)-roscovitine (10 μ M) plus Nutlin-3 (2.5 μ M) was parallel to that displayed by deadly doses of Nutlin-3 (Fig. 4). This fact suggests that the apoptotic mechanisms triggered by the combined therapy are closer to those derived from Nutlin-3 than to those due to (R)-roscovitine.

Nutlin-3 acts as a p53 activator and p53-induced apoptosis is known to partially occur through mitochondrial outer membrane permeability transition [17, 18]. Bcl-XL is a protein of the Bcl-2 family known to exceed Bcl-2 in its ability to neutralize mitochondrial-mediated apoptosis. For instance Bcl-XL overexpression in SH-SY5Y cells, but not Bcl-2 overexpression, is able to counteract staurosporine-induced apoptosis [16]. We therefore investigated the effect of Bcl-XL overexpression on the apoptotic process triggered by (R)-roscovitine, nutlin-3 and the combined treatment. SH-SY5Y cells overexpressing Bcl-XL and empty vector transfected controls were used [16]. As a functional control, we first verified that these Bcl-XL overexpressing cells were resistant to staurosporine-induced apoptosis (Fig. 5A). We further confirmed that, as previously reported [11], neither Bcl-2 nor Bcl-XL overexpression were able to protect SH-SY5Y cells from apoptosis triggered by racemic roscovitine or (R)-roscovitine (Fig. 5A).

We next tested the effect of Bcl-XL overexpression on nutlin-3 induced apoptosis. Bcl-XL overexpression was found to protect SH-SY5Y cells from the apoptotic consequences of a 48 hr treatment with a lethal concentration of nutlin-3 (Fig. 5B). This was not an unexpected finding given the mitochondrial mechanisms of apoptosis triggered by the p53 protein. When 10 μ M (R)-roscovitine + 2.5 μ M nutlin-3 were tested on the pcDNA3/Bcl-XL transfected cells an increase of cell viability was also found (Fig. 5B). The combined treatment and 10 μ M nutlin-3 alone caused a similar level of cell death. Facing these apoptotic stimuli (combination or nutlin-3 alone), the extent of cell protection by Bcl-XL

overexpression was also remarkably similar (Fig. 5B). This result provides additional and relevant clues concerning the nature of the synergy between (R)-roscovitine and nutlin-3. The apoptotic pathway underlying the combined treatment behaves identically to the pathway engaged by lethal concentrations of nutlin-3 alone. Therefore we propose that (R)-roscovitine acts by sensitizing SH-SY5Y cells to the nutlin-3 action on p53.

To further support this conclusion, we explored how these drug treatments were impinging on p53 protein and its transcriptional function. We analyzed the cell content of p53 and two of its transcriptional targets, HDM2 and p21^{Cip1}, by SDS-PAGE followed by Western blotting (Fig. 6). A 24 hr treatment with (R)-roscovitine (10 μ M) was sufficient to induce the stabilization of p53 protein but not apoptosis. In coherence with its reported mode of action, nutlin-3 stabilized p53 and increased HDM2 and p21^{Cip1} protein content in a dose-dependent manner. Interestingly, the combination of 10 μ M (R)-roscovitine + 2.5 μ M nutlin-3 led to an increased expression of p53, HDM2 and p21^{Cip1} similar to that observed with 10 μ M nutlin-3 alone. This result provides a clue for further studies on the molecular mechanisms causing the synergy between (R)-roscovitine and nutlin-3.

DISCUSSION

In this report we have shown (R)-roscovitine synergizes with nutlin-3 at inducing apoptosis in SH-SY5Y, IMR-5 and IMR-32 cell lines derived from human neuroblastoma. In SH-SY5Y cells, Bcl-XL overexpression experiments and protein analysis of p53 stability and transcriptional functions allow us to propose that (R)-roscovitine merely acts by increasing the cellular responses to nutlin-3, namely activation of p53, its downstream targets and apoptosis induction. Our results are consistent with the reported mode of action of nutlin-3, i.e. the inhibition of the interaction between p53 and HDM-2, leading to a stabilization of p53. Apoptosis in response to p53 is known to occur by multiple simultaneous mechanisms, mitochondrial and non-mitochondrial, transcriptional and non-transcriptional [17, 18]. Bcl-XL protein is well known by its ability to block mitochondrial mediated apoptosis and,

consequently, its overexpression in SH-SY5Y provided a partial protection from nutlin-3/p53 mediated apoptosis.

Our results suggest that the synergism between nutlin-3 and (R)-roscovitine in inducing apoptosis relies on the potentiation of nutlin-3 actions by (R)-roscovitine. What is the molecular mechanism involved in this potentiation? (R)-roscovitine is a CDK-inhibitory drug that has demonstrated to be very selective for CDKs in biochemical assays [19-21]. How could the inhibition of one or more CDK contribute to the stability of the p53 protein? The activation of p53 has been reported in cells challenged with roscovitine and this might be due to a decrease in HDM-2 protein [22]. However, in SH-SY5Y cells exposed to sublethal concentrations of (R)-roscovitine, we have observed a slight increase, rather than a decrease, in HDM-2 level (Fig. 6). The pharmacological inhibition of CDK9 has proven to promote the nuclear accumulation of p53 without diminishing MDM-2 transcription [23]. Furthermore, the inhibition of CDK2 has been reported to activate an intra-S-phase checkpoint that leads to an ATM/ATR-dependent phosphorylation and activation of p53 protein [24]. Interestingly, CDK9 and CDK2 are among the most sensitive targets of (R)-roscovitine.

The apoptotic process triggered by either racemic roscovitine [11] or (R)-roscovitine (this report) is insensitive to the overexpression of either Bcl-XL or Bcl-2. In contrast Bcl-XL overexpression protects SH-SY5Y cells from nutlin-3 triggered, p53 mediated apoptosis. Taken together, these data suggest only a minor, if any, role for p53 in the apoptotic process triggered by roscovitine itself. In other words, the mitochondrial mechanisms activated by p53 to cause apoptosis does not seem to be involved in the apoptotic process induced by CDK inhibitory drugs like (R)-roscovitine, racemic roscovitine or olomoucine alone.

A deficit in p53 function is found in more than 50% of all human cancers. However in many cancer types, a functional p53 system is present. This is the case of human neuroblastoma tumors and cell lines, like SH-SY5Y [15]. Therefore, nutlin-3 constitutes a promising drug for the treatment of this childhood malignancy. The synergism we report here

is found in three different cell lines derived from human neuroblastoma. This fact suggests that a low dose of (R)-roscovitrine (or other CDK inhibitors) might allow to reduce the dose of nutlin-3 (or related compounds) without reducing its therapeutic efficacy against neuroblastoma tumors. Finally, Bcl-2 or Bcl-XL overexpression and the subsequent resistance to apoptosis, are associated with a reduced response to chemotherapy. The fact that Bcl-2 and Bcl-XL fail to block the apoptotic process triggered by (R)-roscovitrine, suggests that resistances could be counteracted in a combination therapy scenario by simply raising the (R)-roscovitrine dosage. In addition to the implications for the treatment of neuroblastoma, our results further confirm the interest of combining CDK inhibitors with other drugs acting on very different targets.

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REFERENCES

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Figure Legends

Figure 1. (R)-roscovitine (R) and nutlin-3 (N) display a pharmacological synergism at inducing cell death. SH-SY5Y cells were treated for 48 hr with (R)-roscovitine at sublethal (10 μ M; black bar) or lethal (50 μ M, white bar) concentrations. Cells were also treated for 48 hr with nutlin-3 at sublethal (2.5 μ M, black bar) or lethal (10 μ M, white bar) concentrations. In parallel, cells were also challenged with 10 μ M (R)-roscovitine plus 2.5 μ M nutlin-3 (R+N). **(A)** Determination of cell viability was performed by the MTS reduction assay. Mean $\pm$ SEM of three independent experiments with six independent measures per experiment. **(B)** Determination of cell death was performed by the LDH release assay. Mean $\pm$ SEM of three independent experiments with three independent samples per experiment.

Figure 2. The cell death process triggered by the combination of (R)-roscovitine and nutlin-3 is apoptotic. SH-SY5Y cells were treated with 10 μ M (R)-roscovitine (R), 2.5 μ M nutlin-3 (N), both (R+N) or left untreated (U) for 24 hr. Apoptotic nuclei were observed by fluorescence microscopy following bisBenzimide staining. Apoptotic nuclei can be easily identified by their brightness and fragmentation in the R+N sample. Scale bar = 50 μ M.

Figure 3. (R)-roscovitine and nutlin-3 synergize at inducing effector caspase activation in three different cell lines derived from human neuroblastoma. SH-SY5Y cells were treated for 12 hr with 10 μ M (R)-roscovitine (R), 2.5 μ M nutlin-3 (N) or their combination (R+N). IMR-5 and IMR-32 were treated for 12 hr with 12.5 μ M (R)-roscovitine (R), 2.5 μ M nutlin-3 (N) or their combination (R+N). Effector caspase activation (DEVDase activity) is expressed in arbitrary fluorescent units. Mean $\pm$ SEM of six independent determinations are shown.

Figure 4. The cell death kinetics triggered by the combination of (R)-roscovitine and nutlin-3 differs from that of (R)-roscovitine and mimics the Nutlin-3 one. SH-SY5Y cells were treated with lethal concentrations of (R)-roscovitine (R) (37.5 μ M) and nutlin-3 (N) (5 μ M). Cells were also treated with a combination of both drugs (R+N), but at their sublethal concentrations (10 and 2.5 μ M, respectively). Cell viability was determined by the MTS reduction assay following 24 hr (black bars) and 48 hr (white bars) treatment. Cell death (%) = 100 - cell viability (%). Mean $\pm$ SEM of at least five independent experiments with at least three independent determinations per experiment are shown.

Figure 5. Effects caused by Bcl-XL overexpression on SH-SY5Y cells treated with (R)-roscovitine (R), racemic (R+S)-roscovitine (R/S), staurosporine (Sts), nutlin-3 (N) and the R + N combination. Concentrations are stated in the figure. Cell viability was determined by the MTS reduction assay after 24 hr (**A**) and 48 hr of treatment (**B**). SH-SY5Y cells transfected with the pcDNA3/Bcl-XL construct (white bars) and the pcDNA3/empty vector (black bars). Mean $\pm$ SEM of at least three independent experiments with at least three independent determinations per experiment.

Figure 6. Effects of (R)-roscovitine on nutlin-3 induced p53 stabilization and transcriptional activation. SH-SY5Y cells were treated for 24 hr with 10 μ M (R)-roscovitine (R), 2.5 μ M and 10 μ M nutlin-3 (N), 10 μ M (R)-roscovitine + 2.5 μ M nutlin-3 (R+N) or left untreated (U). Extracts were obtained and analyzed by SDS-PAGE followed by Western blotting with antibodies directed against the proteins indicated in the figure. Tubulin levels were used as loading controls.

Table 1

The pharmacological synergism between (R)-roscovitine and nutlin-3 is detected in three different cell lines derived from human neuroblastoma.

Cell line	(R)-roscovitine	nutlin-3	(R)-roscovitine + nutlin-3
SH-SY5Y	80.51 ± 1.70 %	65.46 ± 0.58 %	26.57 ± 1.13 %
IMR-5	79.07 ± 0.28 %	72.26 ± 1.21 %	26.84 ± 0.70 %
IMR-32	82 ± 1.01 %	71.07 ± 1.04 %	19.00 ± 1.28 %

(R)-roscovitine was used at a concentration 10 μ M in SH-SY5Y cells and 12.5 μ M in IMR-5 and IMR-32 cells. Irrespective of the cell line or treatment, nutlin-3 concentration was 2,5 μ M. Cell viability was determined by the MTS procedure after 48 hr of treatment. The percentage of cell viability is referred to those of control untreated cells. Mean $\pm$ SEM of three independent experiments with six independent measures per experiment is reported.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.