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Torin 1, TOR Inhibitor Enhances Cellular Proliferation in NT-1 Tobacco Suspension Cell Cultures

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Abstract: Torin 1 is an ATP-competitive TOR inhibitor which inhibits the signaling of TOR and S6K kinase in mammals and plants. The objective of this research is to determine the effect of Torin 1 in a relatively simple and homogeneous plant system such as the NT-1 tobacco suspension cell cultures. Cultures of NT-1 cells were tested with 5, 50, 150 and 250 nM of Torin 1. During kinetics growth of NT-1 tobacco suspension cell cultures, 150 and 250 nM Torin 1 inhibits the early growth and later enhanced the cellular proliferation during exponential growth by means of an increased expression of *E2F1* and *cyclin B*. Furthermore, Torin 1 stimulates the growth of NT-1 cells during log phase with small shaped cell, characteristic of tobacco suspension cell cultures with high mitotic activity.

Keywords: Torin 1; TOR, S6K; proliferation; NT-1 tobacco cells

1 Introduction

The target of rapamycin (TOR) is a conserved eukaryotic phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) that regulates growth and metabolism in response to environment [1-3]. TOR is part of a two-protein complex TORC1 and TORC2, the first is sensitive, while TORC2 is insensible to rapamycin [4, 5]. In mammals TOR activity is regulated by insulin and insulin-like growth factors (IGF) via the PI3K-TOR-S6K route; also TOR is activated by nutrients and ATP [6]. The genome of *Arabidopsis*, as that of other eukaryotes, contains a single copy of TOR, which causes embryonic lethality when it is mutated [7]. TOR level correlates with plant size indicating its central role in plant development [3]. Also, this kinase regulates processes like translation, ribosome biogenesis, actin dynamics, production of reactive oxygen species (ROS) and autophagy [8]. The activity of TOR has been investigated in mammals mainly via its inhibition by rapamycin, a macrolide with therapeutic applications as antifungal, immunosuppressant, and in cancer therapy [9]. The FKBP12-rapamycin duo binds to the called FRB (FKBP12-Rapamycin Bind) domain of TOR therefore creating a ternary complex that inhibits TOR kinase activity. Studies of TOR kinase have increased over the last years and limitations of rapamycin based clinical approach have forced the development of ATP-competitive TOR inhibitors that were called as TOR is (active site TOR inhibitors) like Torin 1, brought new tools to study the TOR pathway [10]. While rapamycin resistance has been reported in some higher plant, there are other plants, e.g., corn and green algae such as *Chlamydomonas reinhardtii* [11,12], which are sensitive to its effect. Recently, *Arabidopsis* sensitivity to high concentrations of rapamycin (100-1000 nM) has been reported; the authors suggest that the variable effects on insensitivity to rapamycin in various biological systems could be linked to variations in the amount of FKBP12 protein, which could explain the controversy around the resistance of certain plants to this inhibitor [13]. Several reports concerning at use of Torin 1 in plants, showed a strongly inhibit growth of a large variety of plants like *Arabidopsis*, potato, tomato, rice, Lotus, millet and Nicotiana and proliferation in both green algae and

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diatoms (*C. reinhardtii, P. tricornutum*) [14-20]. It has also been observed that 200 nM Torin 1 inhibits the phosphorylation of TOR and S6K1 in cellular lines of *Arabidopsis* overexpressing TAV viral factor [21]. In mammals, Torin 1 inhibition affects the proliferation, and protein synthesis and cause an increase in the eccentricity of the nucleus at concentrations of 50 and 250 nM [10,22]. In plants, the respond of TOR at environment, hormones and stress has recently been demonstrated [23]. For example, Li et al., reported that through TOR, the cellular division of apical and radicular meristem of *Arabidopsis* is reactivated by light and glucose respectively [24]. For this reason, it is important to know how TOR inhibitors regulated cellular processes such as the cell cycle. The cell cycle is a highly regulated event that generates two daughter cells from a progenitor cell and consists of four consecutive phases: G1, S, G2 and M. The remarkable genetic conservation of the cell cycle in eukaryotes has been confirmed with studies in the genome of *Arabidopsis thaliana* [24,25]. The progression through the cell cycle in plants is regulated by the heterodimeric kinases cyclins and the catalytic subunits cyclin dependent kinases (CDK) [26]. Cyclin B participate in the G2/M progression. Cyclins B and D expression depends mainly on auxins and cytokinins, growth conditions and development [27]. The E2F family transcription factors also play an important role in cell cycle progression. The *Arabidopsis* genome contains E2Fa, E2Fb and E2Fc factors that require dimerization with DPa or DPb proteins [28]. The most widely used plant cell lines for recombinant biopharmaceutical production are those derived from tobacco (*Nicotiana tabacum*), such as cultivars BY-2 (*N. tabacum* cv. Bright Yellow 2) cells and NT-1 *(N. tabacum-1*) cells. They have appealing features, including being fast-growing, robust and able to readily undergo *Agrobacterium*-mediated transformation and cell cycle synchronization [29,30]. The tobacco BY-2 cells have also been utilized across a wide spectrum of plant biology, in particular this cell line has contributed significantly to molecular biology studies of the plant cell cycle [31-33]. The present study aims to study the effect of Torin 1, one of inhibitors of second generation of TOR, on plant growth in a relatively simpler system than plants such as NT-1 suspension cultures of tobacco cells.

2 Materials and Methods

2.1 Tobacco-Cell Suspension Culture

Chemicals were purchased from Sigma-Aldrich unless otherwise indicated. NT-1 tobacco cells were cultured for 7 d in 4.3 g/L Murashige and Skoog basal medium (Phyto Technology Laboratories, USA), pH 5.8, supplemented with 3% Sucrose, 1 mg/L thiamine, 100 mg/L myoinositol, 0.2 g/L KH_2PO_4, and 0.2 mg/L 2,4-D at 25°C under constant agitation (100 rpm) in the dark. Every 7 days the cells were subcultured in fresh medium as described above.

2.2 NT-1 Tobacco-Cell Growth with Torin 1 Inhibitor

Previously washed NT-1 tobacco cells in stationary phase (day 7) were inoculated (3.5 mL) in 50 mL of fresh MS medium supplemented with Torin 1 inhibitor (LC Laboratories) at concentrations of: 0, 5, 50, 150, and 250 nM. Daily, 3-mL aliquots were taken to evaluate the growth by dry weight and packed-cell volume (PCV).

2.3 Mitotic Index

Before calculating the mitotic index (MI), tobacco cells were fixed with 4% paraformaldehyde and 50% glutaraldehyde during 12 h at 4°C. MI percentage was calculated at day 6. 0.5 mL of log-phase cells were stained with 10 mg/mL 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) in Phosphate Buffered Saline (PBS) for mitotic figure counts (% MI = Cells undergoing mitosis/500 counted cells x 100). Cells were observed under a Nikon fluorescence microscope with a 20X objective. The cellular size was calculated with DAPI-stained cells, which were measured to calculate the length/width ratio with ImageJ.

2.4 Semi-Quantitative RT-PCR for Cell-Cycle Regulator Genes

RNA extraction was performed with NT-1 cells in log phase (day 6) supplemented with 5, 50, or 150 nM of inhibitor Torin 1 [34]. Semi-quantitative RT-PCR analysis (Kit SuperScript III ™ One-Step RT-

PCR System with Platinum® *Taq* DNA polymerase, Invitrogen) of *Cyclin B* (Access number Gen bank D89635.1), *E2F* (AB025347) and *Actin* (X63603) was carried out. For this purpose, 0.64 μg/μL of total RNA was subjected to RT-PCR with the aforementioned kit in a Techne TC-3000 Thermal Cycler (Barloworld Scientific, USA), in amplification conditions optimized for each gene (Tab. 1). The mRNA expression level was calculated relative to actin expression by densitometry using ImageJ.

2.5 Statistical Analyses

Basic statistical parameters and analyses of variance (ANOVA) were performed using the commercial statistical software Minitab17. Differences with P values of ≤ 0.05 were considered statistically significant.

Table 1: Oligonucleotide primers of cell-cycle regulator genes used in semi-quantitative RT-PCR

Target gene	Description	Sequence (5´-3´)	Orientation	Annealing temperature (°C)	Reference
E2Fb	Transcription factor	CAAATTACAAACAGGGAGTTGGTGC	Forward	68	This study
		CTCCTTTGTGGATAATCAACAGCCT	Reverse		
Cyc B	Cell cycle protein	ATAACGAGGGGCTTTTGTGC	Forward	60	[35]
		CTTTTCTCCATTCCCAACACCT	Reverse		
Actin	Cytoskeleton protein	CCTCTTAACCCGAAGGCTAA	Forward	55	This study
		GAAGGTTGGAAAAGGACTTC	Reverse		

3 Results

Due to the importance of TOR in plant development and its different responses to drugs that inhibit its activity, the effect of different concentrations of Torin 1 was tested in growth kinetics of NT-1 cultures. Cultures with 150 and 250 nM of Torin 1 inhibited dry-weight growth until day 4 (the start of log phase) and showed an increase in biomass from day 6 onward (Fig. 1(A)); while cultures with 5 and 50 nM of Torin 1 showed a tendency to increasing dry weight from day 2 onward, maintaining this tendency for the duration of the growth kinetics. With 150 and 250 nM of Torin 1**,** the PCV kinetics showed faster growth over the course of the experiment (Fig. 1(B)). The Mitotic Index (MI) was evaluated to correlated with proliferation (Fig. 2(A)), showing a decrease (by 30%) only with 50 nM of Torin 1. Cells supplemented with 150 and 250 nM showed a 25% MI increase, which correlates with the increase in biomass and PCV (Figs. 1(A) and 1(B)). The Torin 1 effect on cell size was evaluated because of the PCV increased values (Fig. 1**)**. The calculated lengh/width ratios show that cultures supplemented with 150 and 250 nM of Torin contained smaller cells (Fig. 2(B)), besides, the cells had a clearly more rounded shape than that observed with the other treatments.

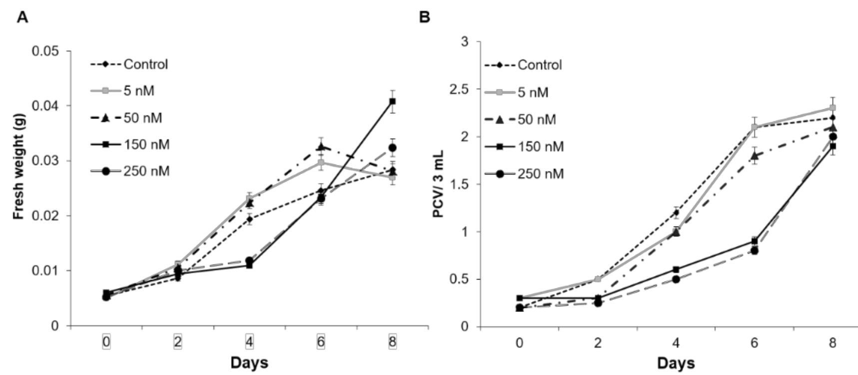

Figure 1: Torin 1 effect on NT-1 culture growth. Previously washed NT-1 cells in stationary phase (day 7) were inoculated (3.5 mL) in 50 mL of fresh MS medium containing different Torin 1 concentrations: 0, 5, 50, 150, and 250 nM. Daily, 3-mL aliquots were taken, kinetics were evaluated by A) dry weight and B) Packed-cell volume. Tukey P = 0.05; n = 2. The experiment was repeated twice with similar results

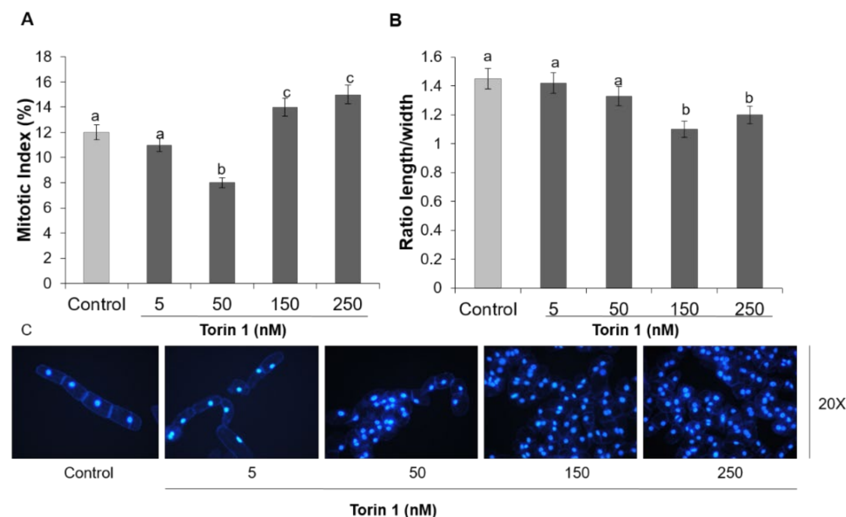

Figure 2: Torin 1 stimulates cellular proliferation of NT-1 cultures. A) Mitotic Index (MI) percentage. Day 6 log-phase cells were stained with DAPI for mitotic figure count (% MI = Cells undergoing mitosis/500 counted cells x 100). Cells were observed under a Nikon fluorescence microscope with a 20X objective. B) Cell size. DAPI-stained cells were measured to calculate their length/width ratios with ImageJ C) DAPI-stained NT-1 cells in log phase.Tukey. $P \leq 0.05$; n = 4

The expression of cell-cycle regulators like *E2F* and *Cyclin B* was evaluated because cell proliferation was observed with high concentrations of Torin 1 from day 6 onward (Fig. 3). The expression of both genes was stimulated.

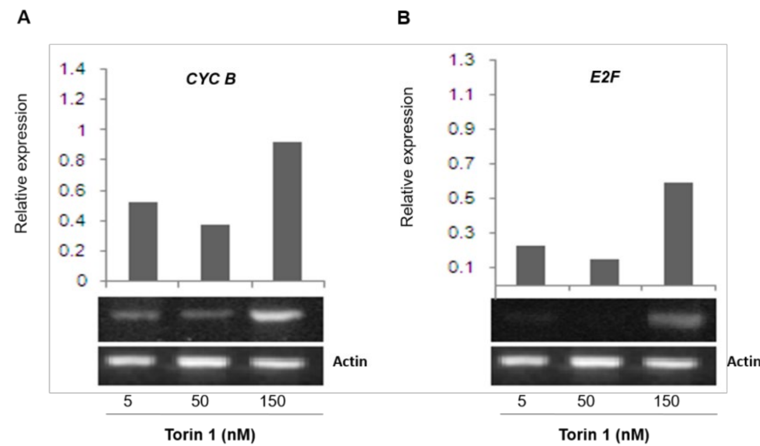

Figure 3: Torin 1 effect cell-cycle regulator genes. RT-PCR semi-quantitative analysis (SuperScript III ™ One-Step RT-PCR System with Platinum® Taq DNA polymerase) of *Cyclin B* (Access number Gen bankD89635.1), *E2F* (AB025347) and *Actin* (X63603) was performed with NT-1 cells in log phase (day 6), supplemented with 5, 50, and 150 nM of Torin 1. The pictures show amplification products of each gene in a 1% agarose gel. The experiment was repeated twice with similar results

4 Discussion

NT-1 cultures exhibit a growth kinetics consisting in lag phase, exponential-growth phase and stationary phase with lower mitotic activity [36]. As far as we know, the long-term effect of Torin 1 on a specific system has not been reported. In this study, we observed that the inhibitor stimulates the growth of NT-1 cultures during log phase, which can be related to the decreased levels of PCV, possibly due to an increase in mitotic activity (Fig. 1). It has been reported that BY-2 cultures show proliferation cells of small size in the population [36]. Torin 1 has been characterized as a highly selective inhibitor of TOR-S6K signaling in mammals and *Arabidopsis* [10]. We observed that 150 nM of Torin 1 reduced the phosphorylation of S6, previously stimulated with Insulin (data not shown), the results suggest that it might stimulate cell proliferation during exponential growth by an increased expression of *E2F1* and *cyclin B*. In mammals, 250 nM of Torin 1 completely inhibit proliferation and arrested the cell cycle in G1/S phase, showing smaller cells than at concentration as 50 nM Torin 1 [10].

This small cell shape is characteristic of suspended tobacco cells with high mitotic activity. Animal cells treated with 250 nM showed an altered nucleus eccentricity, which causes a change from round to elongated shape, regulated by the *LIPIN-1* gene [22].

The expression of both genes *E2F1* and *cyclin B* was stimulated, which could explain the increase in MI and biomass in NT-1 cultures (Figs. 1 and 2), contrasting with the activity reported in mammals, where 250 nM of Torin drastically suppressed cell proliferation by reducing the quantity of cyclin D [10]. The stimulation of the cellular proliferation of NT-1 cultures by Torin 1 was really an unexpected fact, because we do not know to date that any TOR inhibitor stimulates growth or cell division. The mechanisms of how Torin 1 stimulates cell proliferation in NT-1 cultures could involve a crosstalk between different branches of the TOR network, that act like a negative feedback loops, where the targets downstream become regulators upstream of the TOR pathway [37]. This type of regulation shows the importance of characterizing cell development or metabolic status of the model studied when trying to decipher the role of TOR pathway [6].

5 Conclusion

Studies in many plant species have shown that TOR plays an important role in the regulation of growth. Therefore, the detailed relationship between proliferation and inhibition by drugs such as Torin 1 in cell

cultures should be investigated, because there are still many unknown mechanisms of regulation of TOR in plants and mammals. Torin 1 applied to cell cultures of tobacco suspension NT-1, inhibits early growth and then rises cell proliferation by means of an increased expression of cell cycle regulators: *E2F1* and *cyclin B*. Then, how Torin 1 stimulates cell proliferation in NT-1 cultures making this an important subject of study, to better understand the role of TOR in plants.

Conflict of interest: All the researchers listed as authors of the current study declare that there is no conflict of interests regarding the publication of this manuscript.

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