Original Article

Inhibition of mTORC1 by SU6656, the Selective Src Kinase Inhibitor, Is Not Accompanied by Activation of Akt/PKB Signalling in Melanoma Cells

(mTORC1 signalling / Src activity / Akt/PKB / raptor / rictor / HBL melanoma cells)

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Abstract. The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase conserved in all eukaryotes that plays a key role in cell growth and is a central effector of several pathways regulating essential cell functions. Hyperactivation of the mTOR-dependent signalling pathway occurs in many human diseases and may be a selective target for their therapy. However, the dual nature of mTOR, existing in two multiprotein complexes mTORC1 and mTORC2 driven by different feedback loops, decreases the therapeutic effects of rapamycin, the specific mTOR inhibitor. In the present study we demonstrate that the mTORC1 signalling pathway is highly activated in human melanoma cells and that up-regulation of this pathway along with the growth and malignity of these cells could be suppressed by disruption of the Src activity. SU6656, the selective inhibitor of the Src kinase activity, decreased up-regulation of the mTORC1 signalling and moreover, unlike rapamycin, it did not induce the activation of Akt/PKB and its downstream targets in HBL melanoma cells. The Src protein was found to be associated with raptor in the mTORC1 complex immunoprecipitated from these cells, suggesting that the Src activity might be a new attractive target for monotherapeutic inhibition of the up-regulated mTORC1 signalling pathway.

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Abbreaviations: DMEM – Dulbecco's Modified Eagle's Medium, FCS – foetal calf serum, mTOR – mammalian target of rapamycin.

Indroduction

Protein kinase cascades in cell signalling pathways are very often deregulated in human diseases and thus represent a valuable target for therapy. One of the most important protein kinase cascades that play a key role in many aspects of cellular physiology is the signalling pathway in which growth-regulating signals from outside and within the cell are integrated through serine/threonine protein kinase mammalian target of rapamycin (mTOR). The mTOR signalling network is also influenced by nutrient and energy levels and is implicated in the regulation of transcription, autophagy, ribosome biogenesis, mRNA turnover, cytoskeletal organization and selective mRNA translation (Wullschleger et al., 2006). mTOR activation occurs downstream of PI3K-Akt signalling in this pathway. mTOR is negatively regulated by suppressor protein tuberous sclerosis complex (TSC1/2) comprising hamartin (TSC1) and tuberin (TSC2). Tuberin is directly phosphorylated by Akt/PKB, which disrupts the TSC1/2 complex and stimulates mTOR activity. mTOR exists in two multiprotein heterometric complexes, mTORC1 and mTORC2. More intensively studied signalling complex mTORC1 controls selective translation of mRNA (Ma and Blenis, 2009) and consists specifically of mTOR, Gβl and raptor (regulatory associated protein of TOR). The mTORC2 complex has been shown to particularly contain mTOR, Gβl and rictor (rapamycin-insensitive companion of mTOR) and controls actin cytoskeleton dynamics (Loewith et al., 2002).

Constitutive activation of the Akt/mTOR pathway in cancer cells supports mTOR as a selective target for cancer therapy, with direct clinical applications. mTORC1 seems to be the only known target of the specific inhibitor of mTOR, drug rapamycin. Treatment with rapamycin inhibits proliferation and may lead to apoptosis, and thus rapamycin and its analogues (rapalogues) are evaluated in clinical trials as important anti-cancer agents. However, rapamycin has been found to enhance Akt/PKB activation in many cell lines, paradoxically activating signalling that reduced the anti-tumor effects of mTOR

inhibition. Akt/PKB activation indicates the existence of a basal mTORC1-dependent feedback mechanism, probably induced by allosteric interactions of rapamycin with mTOR. In this feedback loop, mTOR associated with rictor in the mTORC2 complex directly phosphorylates Akt on serine 473, thereby contributing to Akt activation (Sarbassov et al., 2005). To avoid the events attenuating growth-inhibitory effects of rapamycin, combination therapies with dual inhibitors are in trials (Mallon et al., 2011). Also, a second generation of inhibitors has been recently developed that competitively target the ATP-binding site of the mTOR kinase domain to block the mTOR protein kinase activity in both mTORC1 and mTORC2 simultaneously, though insufficiently (Vilar et al., 2011).

We have previously demonstrated that the activity of non-receptor tyrosine kinase Src is involved in the upregulation of the mTOR signalling pathway (Vojtěchová et al., 2008) in RSV-transformed hamster fibroblasts and is suppressed by SU6656, the specific small-molecular inhibitor of Src kinase activity (Blake et al., 2000). In this study we used the inhibitor SU6656 to analyse the regulatory role of Src in the mTOR-dependent signalling pathway in melanoma cells. We found that the disruption of Src kinase activity by treatment of HBL melanoma cells with SU6656 suppressed mTORC1 signalling in a similar way as rapamycin and moreover, unlike rapamycin, treatment of the cells with SU6656 did not induce activation of Akt and its downstream signalling.

Material and Methods

All general reagents were from Sigma (St. Louis, MO) unless otherwise stated. Protein G-Sepharose and ECL Prime WB detection reagent were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). All primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Cell culture and preparation of cell extracts

Human melanoma cell lines used in this study were described previously (Vachtenheim et al., 2001). HBL cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS), antibiotics, and L-glutamine. Malme 3M, MeWo, SK-MEL-28, SK-MEL-5 cells were grown in Eagle's minimal essential medium containing 10% FBS, antibiotics, L-glutamine, sodium pyruvate and non-essential amino acids. Normal human melanocytes were purchased from Cascade Biologics (Invitrogen, Carlsbad, CA) and maintained in the supplemented 254 medium as recommended by the manufacturer. Cells were brought to quiescence by an overnight incubation with 2% FCS and then treated with the inhibitor rapamycin or SU6656 added to a final concentration of 20 nM and 5 μM (DMSO solution diluted in the medium, $1 : 10^6$), respectively, or DMSO carrier alone for time periods as indicated. The cell lysates were prepared in ice-cold extraction buffer [50 mM TRIS pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 40 mM β-glycerophosphate, proteinase inhibitors aprotinin, leupetin and pepstatin (0.4 μg/ml each), 1 mM PMSF and PhosSTOP (Roche Diagnostic, Mannheim Germany)]. Cell debris was pelleted by centrifugation at 20,000 *g* for 10 min, and supernatants were assayed for protein concentration.

Immunoprecipitation of proteins, Western blot analysis

For immunoprecipitation analyses the cell lysates were prepared in the extraction buffer containing 0.3% CHAPS instead of 1% Triton X-100. Proteins were immunoprecipitated from the cell lysates on Protein G-Sepharose beads, and the resulting immunoprecipitates were used for SDS-PAGE followed by immunoblot analysis as described (Vojtěchová et al., 2008).

Soft agar assay

Approximately 5×10^4 cells were seeded in the upper layer containing 3 ml of DMEM medium, 0.45% agar, 10% FCS supplemented with DMSO or inhibitors in a final concentration of 5 μM SU6656 or 20 nM rapamycin. The media were refreshed every 3–4 days. The colonies were stained with p-indonitrotetrazolium violet, counted, and photographed (magnification × 20) after 24 days.

Proliferation rates

HBL melanoma cells were seeded in triplicate wells, grown for time periods as indicated, fixed and stained with 0.1% crystal violet. The dye was quantified.

Results

Melanoma is a highly malignant skin cancer with multiple genetic defects including activating mutations in the *Braf* kinase gene and alterations in signal transduction pathways regulating proliferation and apoptosis (Zheng et al., 2009). We have found that in several melanoma cell lines (MeWo, Malme 3M, SK-MEL-5, SK-MEL-28, HBL), in contrast to normal human melanocytes, the activity of the mTOR-dependent signalling pathway is highly elevated (not shown). To analyse mTOR signalling in more detail, in this study we used a wild type of melanoma cells, HBL human cells (Herraiz et al., 2012), to avoid any possible misinterpretation of the obtained results due to the effects of BRAF mutations. Interactions between mutant BRAF and Akt/PKB pathway were identified in a subset of melanoma cells harbouring mutant BRAF V600E (Chen et al., 2012).

Elevated Akt/PKB signalling upon rapamycin treatment of melanoma cells

Rapamycin is a well-known specific inhibitor of mTORC1 signalling in many human cancer cells and also in melanoma cells. To examine the effects of rapamycin in HBL cells we analysed changes in the phos-

phorylation level of key effector proteins of the mTORC1 signalling pathway, mTOR, S6 kinase (S6K1) and its specific target, ribosomal protein S6. Phosphorylation of Ser2448 at mTOR decreased in cells treated for 60 min with rapamycin to about 40 % of the total mTOR phosphorylation in untreated control HBL cells. Rapamycin-induced inhibition of mTORC1 downstream signalling was rapid as judged by the increased electrophoretic mobility of protein kinase S6K1 and by the decreased binding of phospho-specific antibodies to Ser235/236 at ribosomal protein S6, as seen in Fig. 1A. Rapamycin treatment for 15 min decreased phosphorylation of the S6 protein to 80 % of the control, untreated cells, and complete inhibition required 30–60 min. Thr1135 phosphorylation of rictor, a component of the mTORC2 complex which was shown to be another target of the mTORC1-dependent kinase S6K1 (Treins et al., 2010), was also strongly inhibited in HBL cells upon treatment of the cells with rapamycin (Fig. 1A).

The positive effect of rapamycin in the suppression of hyperactivity of the mTORC1 signalling pathway has very often been associated with increased activity of Akt/PKB induced by prolonged rapamycin treatment, probably by a feedback mechanism involving mTORC2 (Hresko and Mueckler, 2005; Sarbassov et al., 2005). In HBL cells, rapamycin treatment for 1, 2 and 8 h indeed increased phosphorylation of the regulatory amino acid residue, Ser473, as well as of Thr308 in the Akt molecule, generating completely activated Akt. The increased phosphorylation and thus activation of Akt was associated with increased phosphorylation of Akt substrates, tuberin and MAPK, as compared with control untreated HBL cells (Fig. 1B). The increase in their phosphorylation began after 60 min of rapamycin exposure and remained elevated after 2 h and 8 h treatment. Prolonged rapamycin treatment, however, did not enhance the mTORC1/S6K1 signalling. Suppression of the phosphorylation of mTOR, S6K1, S6 and rictor was not changed upon treatment of the cells with rapamycin for up to 20 h (not shown).

SU6656, the selective inhibitor of Src kinase activity, decreased mTORC1 and Akt/PKB signalling

We have previously identified an important implication of Src tyrosine kinase activity in the regulation of the mTOR signalling pathway in RSV-transformed hamster cells (Vojtěchová et al., 2008). Treatment of v-*src*-transformed cells with SU6656, the specific small-molecule inhibitor of the Src family kinase, resulted in the decreased phosphorylation of signalling proteins in the mTORC1-dependent pathway in v-*src*-transformed cells.

Using SU6656, we now examined a possible role of Src activity in the up-regulated mTOR-dependent signalling in HBL melanoma cells. As seen in Fig. 2A, phosphorylation of mTOR (Ser2448), S6K1, S6 protein (Ser235/236) and rictor (Thr1135) decreased upon treatment of HBL cells with SU6656 in a time-dependent manner, similarly as in the cells that were treated with rapamycin (Fig. 1A). Attenuation of rictor phosphorylation at Thr1135 also by SU6656 is in agreement with the presumed fact that this phosphorylation is catalysed by S6K1 (Julien et al., 2010; Treins et al., 2010).

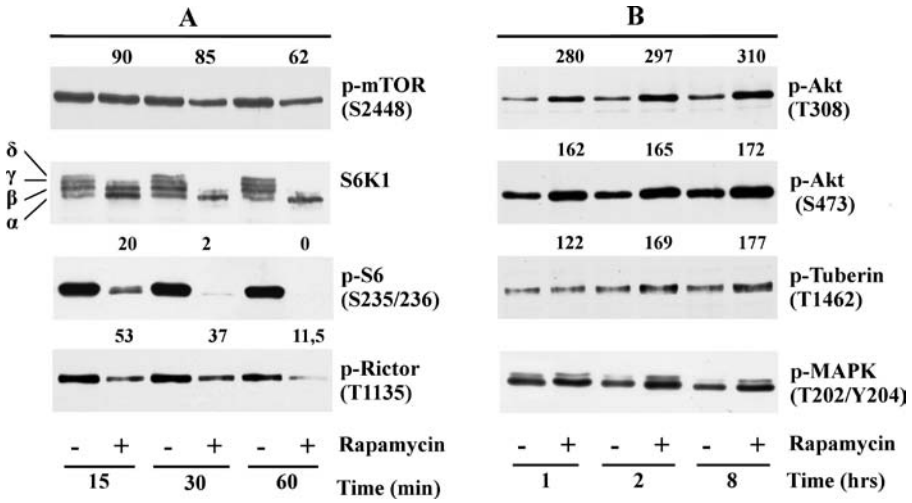

Fig. 1. Phosphorylation of (**A**) mTOR, S6K1, ribosomal protein S6, rictor, and (**B**) Akt, tuberin, MAPK in HBL melanoma cells that were incubated with rapamycin

Crude extracts (15 μg) of HBL melanoma cells that were incubated with rapamycin (20 nM) (+) or with vehicle, DMSO (-), for periods of time as indicated were analysed by Western blotting. Phosphorylation of mTOR, S6 protein, rictor, Akt, tuberin, and MAPK was examined using specific antibodies against phosphorylated proteins. The phosphorylation state of S6K1 is demonstrated by the gel shift method, where the highest phosphorylated form of S6K1 moves in the gel more slowly (γ, δ) than the less phosphorylated (β) or unphosphorylated (α) form of S6K1, as described in Material and Methods. Numbers at the bands express their densitometric evaluation related to their relevant control bands that are taken as 100. Data are the means of at least three independent experiments from which one experiment is depicted.

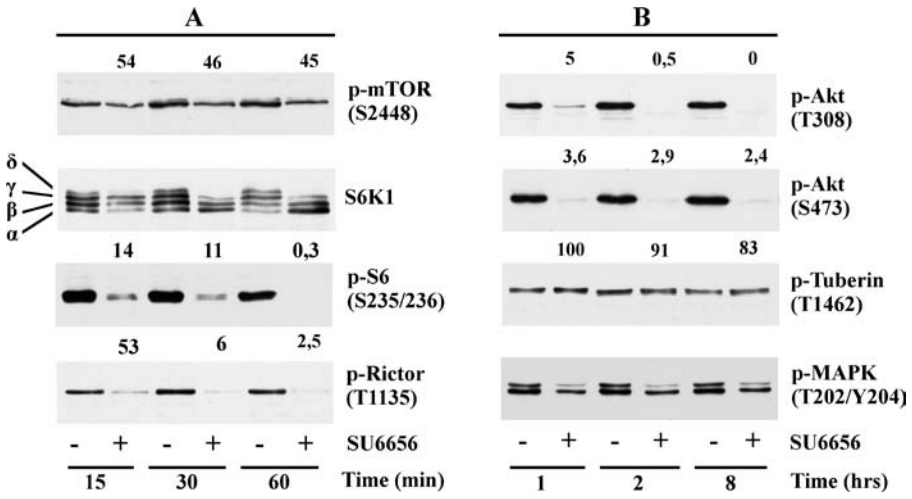

Fig. 2. Phosphorylation of (A) mTOR, S6K1, ribosomal protein S6, rictor, and (B) Akt, tuberin, MAPK in HBL melanoma cells that were incubated with the Src inhibitor SU6656

Crude extracts (15 μg) of melanoma cells HBL that were incubated with the inhibitor SU66S6 (5 μM) (+) or with vehicle, DMSO (-), for periods of time as indicated were analysed by Western blotting. Phosphorylation of mTOR, S6 protein, rictor, Akt, tuberin, and MAPK was examined using specific antibodies against phosphorylated proteins and the phosphorylation state of S6K1 is demonstrated by the gel shift method as described in Fig. 1. Numbers at the bands express their densitometric evaluation related to their relevant control bands that are taken as 100. Data are the means of at least three independent experiments from which one experiment is depicted.

Surprisingly, inhibition of the mTOR signalling pathway by SU6656 was not accompanied by increased phosphorylation and thus activation of Akt and its downstream signalling, as it was seen in HBL cells treated with rapamycin (Fig. 2B). Moreover, incubation of the cells with SU6656 strongly decreased phosphorylation of Akt at Ser473 and Thr308 compared to untreated cells, implying that Akt is a substrate for Src kinase activity (Jiang and Qiu, 2003; Vojtěchová et al., 2008).

Src protein is associated with raptor in mTORC1

To detect possible interactions of Src with the mTOR complexes, we separated raptor, the adaptor protein of mTORC1, and rictor, the adaptor protein of mTORC2, from HBL cells by immunoprecipitation. As seen in Fig. 3A, Src was present only in the immunoprecipitates of raptor, and not in the rictor immunoprecipitates. The association of Src with raptor agrees with the presence of raptor only in the immunoprecipitates of Src from HBL melanoma cells (Fig. 3B).

Cell proliferation and soft agar cell colony formation are suppressed by both SU6656 and rapamycin

Exposure of HBL cells to rapamycin and SU6656 caused inhibition of cell proliferation at concentrations 5 μM SU6656 and 20 nM rapamycin, which are sufficient to profoundly inhibit mTORC1 signalling. The growth inhibition of HBL cells by SU6656 was slightly more effective than by rapamycin (Fig. 4A).

The soft agar assay was used to examine the capability of SU6656 and rapamycin to influence the phenotype of HBL cells. The block of cell colony formation corre-

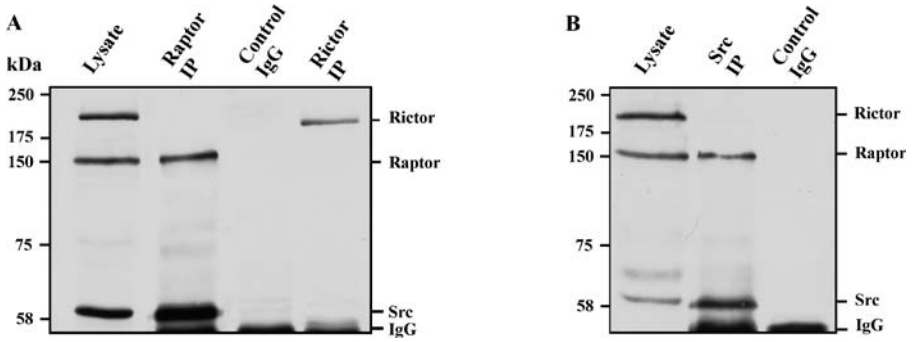

Fig. 3. The presence of Src protein in the raptor complex in HBL melanoma cells

Total cell lysate of HBL melanoma cells (15 μg), (**A**) raptor and rictor complex immunoprecipitated from the cell lysates, and (**B**) Src protein immunoprecipitated from the cell lysate (400 μg) were analysed by Western blotting using specific anti-raptor, anti-rictor and anti-Src antibodies as described in Material and Methods. Two of five independent experiments are depicted.

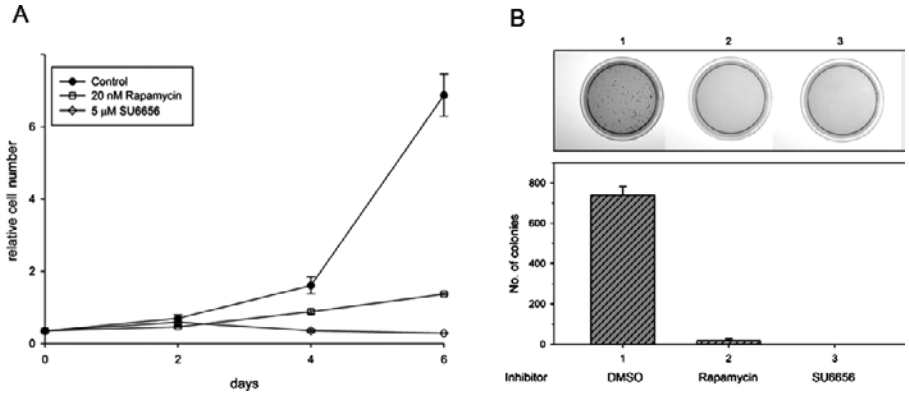

Fig. 4. Proliferation rates (**A**) and soft agar colony formation assay (**B**) of HBL melanoma cells
(A) HBL melanoma cells were grown in the presence of DMSO (●), 5 μM SU6656 (□) or 20 nM rapamycin (◊) for the time periods as indicated. The relative cell number was estimated by staining as described in Material and Methods. The values are means of duplicates ± SE. **(B)** The soft agar assay of the effect of the inhibitors, rapamycin and SU6656, on the malignant properties of HBL melanoma cells was carried out in 60 mm Petri dishes as described in Material and Methods.

lates well with the inhibition of mTORC1 signalling induced by treatment of HBL cells with these inhibitors, indicating relations between malignant growth of cells and the mTORC1 activation. Rapamycin was again less efficient than SU6656 in suppressing malignant transformation of HBL melanoma cells, as shown in Fig. 4B.

Discussion

Elevated tyrosine kinase activity of the protein product of c-*src* protooncogene was found in a number of human tumours and thus Src inhibition has recently become a target for drug therapy. Increased Src expression and activity exists in melanoma cell lines and in melanoma tumours *in vivo* (Barnekow et al., 1987; Homsi et al., 2007). It was shown that Src regulates STAT3, STAT5, βFGF receptors, N-cadherin and β-catenin in melanoma, and their activated forms were detected in both primary and metastatic melanoma in humans. Several melanoma cell lines were exposed to inhibitors of Src tyrosine kinase activity alone or in combination with standard chemotherapy agents (Homsi et al., 2009; Ferguson et al., 2013), although the specificity of these inhibitors was rather wide. Dasatinib, a multi-target tyrosine kinase inhibitor, was the first Src inhibitor approved for use in the treatment of adults with chronic myeloid leukaemia (Eustace et al., 2008). However, the regulatory mechanism of Src is not quite clear.

As it is demonstrated in this paper, disruption of Src kinase activity in HBL melanoma cells by their treatment with SU6656, the selective inhibitor of Src kinase activity, decreased the hyperactivity of the mTORC1 signalling in a similar way as the treatment with specific inhibitor of mTOR, rapamycin. In addition, inhibition of mTORC1 by SU6656 did not cause negative anti-proliferative effects resulting from the activation of Akt/PKB signalling by rapamycin treatment, suggesting that suppression of Src activity may block this feedback mechanism. On the other hand, allosteric changes in the mTOR molecule induced by the inhibitory binding of FKBP 12-rapamycin complex to mTOR probably could result in Akt/PKB phosphorylation by mTORC2.

The presence of Src in the immunoprecipitates of raptor observed in this study indicates that Src may be bound to raptor and that changes in the Src tyrosine kinase activity may thus directly modulate the function of raptor to serve as a scaffold protein of mTOR in mTORC1. There are two key regulatory proteins in the multiprotein mTORC1 and mTORC2 complexes, raptor (Kim et al., 2002) and rictor (Dibble et al., 2009). They are essential for the specific function of each complex, acting as adaptor proteins phosphorylated in multiple sites. This implies that raptor and rictor may be modified by different kinases integrating different signals and playing an important role in the control of mTOR signalling.

Rictor was found to also be a direct target of rapamycin-sensitive complex mTORC1, being phosphorylated by S6K1 at Thr1135 and controlled by the mTORC1 activity. In this study, rictor phosphorylation at Thr1135 was inhibited in HBL cells that were treated by both rapamycin and SU6656. These data are in accordance with the findings that placed rictor downstream to S6K1, indicating a functional relationship between mTORC1 and mTORC2, and support the possibility that rictor may not be required for rapamycin-induced phosphorylation of Akt/PKB by mTORC2 (Boulbes, et al., 2010).

We have reported previously that in v-*src*-transformed cells, the Src kinase activity is essential for the activity of mTOR-dependent pathway in cells expressing hyperactive Src protein, and we suggested that the mTORC1 signalling may be controlled by Src independently of Akt/PKB (Vojtěchová et al., 2008). The fact that basal phosphorylation of Akt/PKB was profoundly decreased by SU6656 treatment suggests simultaneous effects of separate Src inhibition on the activity of Akt/PKB (Jiang and Qiu, 2003; Vojtěchová et al., 2008) and mTORC1 activity, probably preventing activation of Akt/PKB, which can be induced by the rapamycin-mTOR interactions.

Increasing evidence supports mTOR as a selective stable genetic target for direct therapy with clinical applications in human diseases. Data presented here suggest that specific inhibitors of the Src kinase activity might be promising monotherapeutic agents for inhibition of the mTORC1 signalling in melanoma cells and may lead to improvements in the therapy of this troublesome cancer.

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