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Inhibition of mTOR restores cisplatin sensitivity through down-regulation of growth and anti-apoptotic proteins

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Abstract

We show that cisplatin resistance in certain lung cancer cell lines can be reversed through inhibition of mTOR (mammalian Target of Rapamycin). These cell lines appear to possess high levels of phospho-mTOR, phospho-AKT and other growth-related proteins, such as hTERT (human telomerase reverse transcriptase), and Cyclin D3 which decrease upon inhibition of mTOR. Importantly, in one cisplatin resistant cell line which expresses BCL2/BCLxL, treatment with mTOR inhibitor (CCI-779) results in decreased levels of these anti-apoptotic proteins and may contribute to increasing apoptosis. However, continuous exposure to CCI-779 leads to expression of P-gp1 (P-glycoprotein1) and should be taken into consideration in designing clinical trials.

Keywords

Lung cancer; Cisplatin resistance; mTOR; BCL2; BCLxL

1. Introduction

We have previously shown that a majority of cisplatin resistant cell lines possess high levels of elongation factors and ribosomal proteins (Wu et al., 2005). These factors are known to be regulated by mTOR which is a serine/threonine protein kinase that functions as a central modulator of cell growth and proliferation at the level of mRNA translation. The two major down stream effectors of mTOR are 4EBP (eIF4E-binding protein) and P70S6K. Upon phosphorylation, P70S6K up-regulates mRNA translation by stabilizing mRNA containing polypyrimidine tracts at their 5' transcriptional (5' TOP) starting sites (Hay and Sonenberg, 2004). 5' TOP mRNAs primarily encode proteins involved in translation, such as ribosomal protein and elongation factor. Activation of 4EBP1 by mTOR also occurs through direct

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phosphorylation, which results in the release of 4EBP1 from its binding to eIF4E (Hay and Sonenberg, 2004). Once released, eIF4E can then bind to the 5' cap of mRNA and allow initiation of translation (Pause et al., 1994). In this communication, we show that in cisplatin resistant cell lines which are shown to express high levels of mTOR, a number of growth/proliferation and survival related proteins such as cyclin D3, hTERT, and phospho-AKT (pAKT) are elevated. Thus, by inhibiting mTOR using CCI-779, these proteins are inhibited and thereby restoring the cisplatin sensitivity. Indeed, we have previously shown that CCI-779 can reverse cisplatin resistance (Wu et al., 2005). To further determine that the reversal effect is directly related to inhibition of mTOR, we have used siRNA to knock down mTOR and determine if we can also restore cisplatin sensitivity. Additionally, we also have investigated whether inhibition of mTOR alters the anti-apoptosis related proteins such as BCL2 and BCLxL which are expressed in lung cancer cell lines. Recently, it has been shown that mTOR forms two complexes, mTORC1 (mTOR/Raptor) and mTORC2 (mTOR/Rictor). mTORC1 is activated by growth factors and nutrients while mTORC2 complex is known to phosphorylate AKT and hence serves as a feedback mechanism when mTORC1 is inhibited (Corradetti and Guan, 2006). Clinically available mTOR inhibitors (i.e. Rapamycin, CCI-779) only inhibit mTORC1, but not mTORC2 complex. Nevertheless, prolonged exposure of mTOR inhibitor can lead to sequestration of mTOR, therefore lesser mTOR is available to form a complex with its binding partners, either Raptor or Rictor (Sarbasov et al., 2006). Thus, mTORC2 can also be inhibited by this mechanism. We have also evaluated the effectiveness of prolonged rapamycin exposure on the restoration of cisplatin sensitivity.

2. Materials and Methods

2.1. Cell lines

SCLC1 cell line was derived from the bone marrow of SCLC (small cell lung cancer) patient, SCLCSR2 (SR2) cell line is the cisplatin resistant variant derived from SCLC1 which was generated by intermittent exposure to cisplatin as described previously (Savaraj et al., 2003; Xu et al., 1996). This cell line exhibits 20 fold resistance to cisplatin. NSCLCS (non small cell lung cancer) cell line was established from metastatic adenocarcinoma to the brain, NSCLCSC is a cisplatin resistant variant derived from NSCLCS by intermittent exposure to cisplatin. This cell line exhibits 7 fold resistance to both cisplatin and carboplatin (Wu et al., 2005). SCLCRA cell line is the rapamycin resistant variant derived from SCLC1.

2.2. Compounds and antibodies

CCI-779 was kindly provided by Wyeth-Ayrest (Madison, NJ). Cisplatin, VP-16 and adriamycin were purchased from hospital pharmacy (Miami, FL). Cyclin D3, hTERT, phospho-4EBP, AKT, BCL2, BCLxL, Phospho-AKT^{ser473}, mTOR, phospho-mTOR, and phospho-P70S6K were purchased from Cell Signaling Inc (Danvers, MA) Rictor was purchased from Bethyl Lab (Montgomery, TX). Actin was purchased from Sigma (St. Louis, MO).

2.3. Growth inhibitory Assay

Cells were seeded in 24-well dishes at the following numbers per well: 8×10^4 transfected SCLCSR-2, 1.5×10^4 SCLC, and 4×10^4 NSCLC. Transfected SCLC cells were incubated under 5% CO₂ and 95% air at 37° C for 6 to 8 hr to allow attachment. Various concentrations of drugs were added and cultured as previously described (Wu et al., 2005). At the end of 72h, the live cells were assayed using a Vi-Cell cell viability analyzer (Beckman Coulter, Inc., Fullerton, CA).

2.4. siRNA

Dharmafect 1 transfection reagent was used for transfecting 8×10^5 cells using 100 nM of mTOR-targeted siRNA (AAUCAUCCAAUGGUGAAUAA and AAGCACCGUCUUUGCAAGUCA) or siCONTROL® (Dharmacon Co., Denver, CO). Transfected cells were then used for study of growth inhibition and for Western blot as previously described (Maher et al., 2007).

2.5. Western blot analysis

Cells were seeded at 1×10^5 /ml onto 100 mm dishes, and allowed to attach overnight, then treated with cisplatin, with and without CCI-779. At 24 or 48 hrs, cells were harvested by scraping in RIPA buffer as published previously (Wu et al., 2005). Cell lysis was completed by sonication and centrifugation. The total protein was separated on an SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and immunoblotted with the indicated antibodies. Bands were measured using a molecular imager Chemidoc system with Quality One software (Bio-Rad, Richmond, CA).

2.6. P-gp1 (MDR1) gene expression by RT-PCR

Total RNA extraction was previously described (Hu et al., 2003). The primers were the *mdr1* specific sequences GGAGTGTCCGTGGATCACAAG (nt 1909–1930) and TGTTTCAGGATCATCAATTCTTGT (nt 2218–2241). These primers were selected at regions that are only 36.4 and 37.5% similar to the corresponding region of *mdr-3* cDNA. Thus, they should not recognize the *mdr-3* gene. The resulting PCR product from these primers was 232 bp.

3. Results

3.1. Growth inhibitory effect of CCI-779 on cisplatin resistant cell lines

The ID₅₀ of SCLC and NSCLC treated with cisplatin alone, CCI-779 alone, and in combination are shown in Fig. 1A. NSCLCSC was resistant to CCI-779 with an ID₅₀ of 9.4 µg/ml. Additionally, co-treatment with cisplatin in this cell line results in only slight improvement of the ID₅₀ (3.7 µg/ml to 2 µg/ml). In contrast, the addition of 0.01 µg/ml of CCI-779 did reverse cisplatin resistance in SCLCSR2 (SR2), as indicated by the ID₅₀ that decreased from 2.52 to 0.45 µg/ml (5.6 fold). Note that 0.01 µg/ml CCI-779 was previously shown to yield only 10% growth inhibitory effect in small cell lung cancer cell types and has no apoptotic effect in both parental and cisplatin resistant cell lines (Wu et al., 2005). Thus, inhibition of mTOR does appear to restore cisplatin sensitivity in SCLCSR2 cells, but not in NSCLCSC.

3.2. CCI-779 sensitivity related to growth signal pathways

Our data showed that NSCLCSC and its parental counterpart NSCLCS were resistant to CCI-779 (ID₅₀ of 9.4 µg/ml and 8.0 µg/ml, respectively). In order to investigate why NSCLCS/SC are resistant to mTOR inhibitor, we performed a gene-specific array analysis of signal transduction pathways (data not shown). One of the major differences observed in these two pairs of cell lines SCLC1/SR2 and NSCLCS/SC was the lower levels of PI3/AKT in NSCLCS/SC. Since AKT is known to activate mTOR, we have studied pAKT, pmTOR, and its downstream effectors in these two pair of cell lines. Our result clearly shows that NSCLCS/SC have non detectable levels of pAKT as well as lower levels of pmTOR and its down stream effectors p-P70S6K and 4EBP (Fig.1 B). Although the number of cell lines studied is limited, it appears that activation of PI3/AKT may be the determinant for sensitivity to mTOR inhibitor (Dancey, 2006). It is also noteworthy that these cell lines (NSCLCS/SC) do not express p-GP1 (data not shown) which is another mechanisms of CCI-779 resistance (Arceci et al., 1992).

3.3. Effect of siRNA-mediated mTOR knockdown on cisplatin sensitivity

To further confirm whether mTOR is directly responsible for this reversal of cisplatin resistance, we have designed siRNA directed against mTOR. siRNA-mediated inhibition of mTOR was observed at 48 hr and to a greater extent at 72hr (Fig.2 A). Band density assays of mTOR western blots demonstrate that siRNA against mTOR achieves 85-90% knockdown. The mTOR siRNA-transfected cells also showed a decrease in 4EBP phosphorylation (Fig.2 A), similar to those treated by CCI-779 in our previous publication (Wu et al., 2005). Importantly, a 10-fold decrease (2.5 to 0.25ug/mL) in the ID₅₀ for cisplatin was observed in SCLCSR2 cells transfected with mTOR-directed siRNA. This decrease in ID₅₀ is comparable to that observed with co-treatment of CCI-779. These results confirm that the reversal effect is mediated by mTOR inhibition.

3.4. mTOR inhibition down-regulates growth/proliferation-related proteins in cisplatin resistant cell line SR2

We have found that the majority of cisplatin resistant cell lines share some common features, such as increased ribosomal protein, elongation factor, hTERT and cyclin D3 (Wu et al., 2005). These proteins are known to relate to cell growth and proliferation (Averous and Proud, 2006; Chen et al., 2003; Fletcher, 2005). To determine whether inhibiting mTOR results in down-regulation of these proteins, we performed immunoblot analyses on these factors. As shown in Fig. 2B line a and b, Cyclin D3 and hTERT are increased after exposure to cisplatin. Conversely, these proteins were found to decrease after the addition of CCI-779. Similar experiments in NSCLCSC cells (which have relatively low cyclin D3) showed no changes with the addition of CCI-779 (data not shown). These data collectively demonstrate that inhibiting mTOR with CCI-779 attenuates the expression of these growth related proteins, which most likely correlates with increased sensitivity to cisplatin.

3.5. mTOR inhibition affects survival and anti-apoptotic proteins

It is known that PI3/AKT is involved in promoting cellular survival pathways by inhibition of apoptosis (Krasilnikov, 2000). We have found that pAKT is significantly increased in the cisplatin resistant cell line (SR2) which is not surprising since this resistant cell activates the PI3/AKT survival pathway to oppose the insult from cisplatin (Fig. 2B line c). Our results demonstrate that pAKT decreased at 24 hr after the addition of CCI-779. This may contribute to the increased sensitivity to cisplatin. The mechanism why CCI-779 decreased pAKT is not known and may be related to lower levels of Rictor (mTORC2) in this resistant cell line (Fig. 2B line d). This complex is known to act as positive feed back to activate AKT when mTORC1 is inhibited (Corradetti and Guan, 2006).

Besides increased pAKT, SR2 also possesses BCL2 and BCLxL, which are known anti-apoptosis proteins. Interestingly, we have found that CCI-779 also inhibits BCL2/BCLxL in SR2 (Fig.2 B line e and f) and increases the expression of BAX/BAK which most likely contributes in increasing apoptosis as measured by cleaved caspase 3 (Fig. 2B line h). In addition, the ratio of BAX/BAK over BCL2 and BAX/BAK over BCLxL are markedly increased (approximately 6 and 16 fold, respectively) when SR2 are treated with CCI-779 and cisplatin (Fig. 2 B line e-g). In contrast, in parental cells there are no discernible changes due to low levels of BAX/BAK. Collectively, inhibition of growth/proliferative protein as well as survival protein and antiapoptotic protein by mTOR inhibitor, CCI-779, is most likely the mechanism of reversal of cisplatin resistance.

3.6. CCI-779 resistance cell line

Recently, Sarbassov et al. have reported that prolonged rapamycin treatment is more effective at inhibiting both mTORC1 and mTORC2. This improved activity is proposed to be through

sequestration of available mTOR, thereby preventing mTOR to form the complex with its binding partners, Raptor and Rictor (Sarbasov et al., 2006). Thus, chronic use of CCI-779 may be more efficient in restoring cisplatin sensitivity. To evaluate this possibility, we continuously exposed SCLC1 cells to low doses of CCI-779 (less than the ID₅₀) for 4 months. Interestingly, these cells (SCLCRA) developed P-glycoprotein1 (P-gp1) expression as detected by RT-PCR (Supplementary Fig.S1). P-gp1 is an ABC transporter which is known to efflux multiple structurally unrelated chemotherapeutic agents, resulting in multi-drug resistance (Choudhuri and Klaassen, 2006). Indeed, SCLCRA exhibited >6 fold resistance to adriamycin and to VP-16, as well as >10 fold resistance to CCI-779. However, these cells only showed 2-fold resistance to cisplatin (Supplementary Fig.S2). This is not surprising since adriamycin and VP-16 are the substrate of P-gp1, but cisplatin is not effluxed by P-gp1 pump. Evidently, CCI-779 is not able to restore cisplatin sensitivity in this cell line. Thus, our data clearly indicate that chronic exposure to CCI-779 results in P-gp1 expression and hence development of multidrug resistance to a variety of chemotherapeutic agents including CCI-779.

4. Discussion

Our results demonstrate that transfection of cisplatin resistant cells with siRNA directed against mTOR restores cisplatin sensitivity which confirms our previous published data that CCI-779, a known mTOR inhibitor, can reverse cisplatin resistance. In this communication, we have also shown that CCI-779 inhibits cyclin D and hTERT which are elevated in cisplatin resistant cell lines. This finding also has been shown by other laboratories (Schmelzle and Hall, 2000; Zhou et al., 2003). Importantly, our data demonstrate that mTOR inhibitor also down regulates pAKT which is increased in cisplatin resistant cell lines. AKT is often activated under insult and/or stress. Activation of AKT will lead to activation of multiple down stream effectors including mTOR to increase the translation of essential proteins for survival. Thus, inhibition of mTOR will turn off the major downstream effector of pAKT. On the other hand, the inhibition of mTOR will lead to the feed back activation of AKT by another mTOR complex (mTOR-Rictor or mTORC2) to overcome this inhibition (Corradetti and Guan, 2006). This feed back mechanism was not obvious in SR2 line and may be due to the fact that our cell lines have low levels of Rictor. In contrast, for SCLC1 which has higher levels of Rictor, the pAKT is higher upon mTOR inhibition. Even though we observed an increase in Rictor levels in SR2 when treated with CCI-779 alone and in combination with cisplatin, the baseline level of Rictor is intrinsically low in this cell line. Therefore, a slight increase (2 fold) may not be able to induce pAKT, whereas in parental cell, there is a 10 fold increase in Rictor which enables them to effectively phosphorylate AKT. Another new finding is that the levels of BCL2/XL are lower in SR2 after the addition of CCI-779 which may result in further enhanced apoptosis. Interestingly, SR2 also possesses higher levels of pro-apoptotic protein BAX/BAK (Fig.2 B line g) when compared with its parental cells SCLC1 while BCL2 and BCLxL are lower. These findings have also been previously reported (Kumar Biswas et al., 2004). It is still unclear why cisplatin resistant cells possess lower levels of BCL2 but higher amounts of BAX/BAK. Our preliminary data showed that SCLC1 has higher levels of Beclin1 than SR2 which may render BCL2 inactive (data not shown). However, the underlying mechanism why cisplatin selectively induces BAX/BAK but repress BCL2/Beclin1 is not known and is currently being investigated in our laboratory.

We have also found that a cell line resistant to CCI-779 expressed very low levels of pAKT as well as pmTOR. This could explain why mTOR inhibition has no effect on this cell line. It is possible that these cells utilize other growth signaling pathways, and the major cisplatin resistant mechanism is due to drug transport. Overall, our findings suggest that lung cancer cells constitutively expressing high levels of pAKT, pmTOR and pP70S6K are more likely to respond to the growth inhibitory effect of CCI-779 as well as its ability in restoring cisplatin sensitivity.

Given that mTOR inhibitors are clinically available, both in oral (RAD001) and I.V. formulations (CCI-779), it may be possible to apply the concept of using mTOR inhibitors to restore cisplatin sensitivity clinically. Therefore, it will be necessary to determine the best dosing regimen to achieve this effect. Although Sarbassov et al. demonstrated that continuous use of CCI-779 is more effective; however, our results indicate that continuous exposure to CCI-779 can lead to expression of P-gp1 which results in multidrug resistance. Therefore, this schedule treatment may not be applicable in the clinical setting. Determining the most appropriate dosing schedule will be the subject of future experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1A

Growth inhibitory effect of cisplatin alone, CCI-779 alone and incombination (ID₅₀)

	Cisplatin(ug/ml)	CCI-779(ug/ml)	Cis(ug/ml)+ CCI (0.01ug/ml)
SCLC1	0.15 ± 0.05	0.07 ± 0.02	0.18 ± 0.02
SCLCSR2	2.52 ± 0.51	0.06 ± 0.01	0.45 ± 0.03
NSCLCS	0.47 ± 0.1	8 ± 0.2	0.23 ± 0.01
NSCLCSC	3.7 ± 0.5	9.4 ± 0.1	2 ± 0.5

Fig.1B **Figure 1.**

Increased AKT and mTOR activity in lung cancer cell lines correlates with increased sensitivity to mTOR inhibition.

(A) ID₅₀ of lung cancer cell lines treated with cisplatin alone, CCI-779 alone and incombination for 72h. Each data point is the average of triplicate samples with S.D.

(B) Immunoblots of pmTOR, p-P70S6K, and p4EBP in different lung cancer cell lines. Overall, SCLCs are shown to express higher level of pmTOR, p-P70S6K, and p4EBP when compared with NSCLCs.

Fig.2A

Immunoblot of SCLCSR-2 before and after transfected with siRNA directed against mTOR

- 1:SR-2 control
- 2:SR-2 transfected with siRNA (48hrs)
- 3:SR-2 transfected with siRNA (72hrs)
- 4:SR-2 transfected with scramble sequence

Figure 2.

mTOR inhibition affects survival and anti-apoptotic proteins.

(A) Western blot analysis of SR2 treated for 48 hr and 72 hr (Note the band intensity of p-mTOR as well as p4EBP is markedly decreased at 72 hr) confirming the effectiveness of siRNA in down-regulating mTOR.

(B) Immunoblots of growth/proliferation and apoptotic protein in SCLC1 and SCLCSR2 treated with 0.5 μ g/mL and 5 μ g/mL of cisplatin, respectively. The combination of cisplatin and CCI-779 showed a decrease intensity of cyclin D3, hTERT and pAKT expressions in SCLCSR2 but not in SCLC1. Rictor expressions are much less in SCLCSR2 with minor changes upon drug treatment. Note the CCI-779 induced cleaved caspase-3 when combined

with cisplatin is mediated by an increase in the ratio of BAX/BAK : BCL2 (6 folds), as well as BAX/BAK : BCLxL (16 folds). The number above each lane depicted the changes from its control which was arbitrarily set at 1. All the band intensity was normalized with actin. (Experiments were all done at 24hr time point)