Original Articles

Pre-clinical evaluation of AZD-2014, a novel mTORC1/2 dual inhibitor, against renal cell carcinoma

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Abstract

Here we found that dual mTORC1/2 inhibitor AZD-2014 significantly inhibited RCC cell survival and growth, with higher efficiency than conventional mTORC1 inhibitors rapamycin and RAD001. RCC cell apoptosis was also induced by AZD-2014. AZD-2014 disrupted mTORC1/2 assembly and activation, while downregulating HIF-1α/2α and cyclin D1 expressions in RCC cells. Meanwhile, AZD-2014 activated autophagy, detected by p62 degradation, Beclin-1/ATG-5 upregulation and light LC3B-I/-II conversion. Autophagy inhibition by pharmacologic or siRNA-based means increased AZD-2014 activity in vitro, causing substantial RCC cell apoptosis. In vivo, AZD-2014 was more efficient than RAD001 in inhibiting 786-O xenografts and downregulating HIF-1α/2α or p-AKT (Ser-473). Finally, AZD-2014’s activity in vivo was further enhanced by co-administration of the autophagy inhibitor 3-methyladenine. We provide evidence for clinical trials of using AZD-2014 in RCC treatment.

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Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of all human cancers, and its incidence is steadily rising around the world [1]. More than half of RCC patients are found at advanced stages with local or systemic metastasis, resulting in poor prognosis [1]. Chemotherapy, hormonal therapy or radiation are considered to be of only limited value probably due to pre-existing or acquired resistance. Thus, the search for novel and more efficient anti-RCC agents is important and urgent [2,3].

Mammalian target of rapamycin (mTOR) is over-expressed and/or hyper-activated in RCC, representing a valuable therapeutic target [4]. mTOR exists in at least two functionally distinct multi-protein complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR, Raptor, mLST8, as well as recently identified PRAS40 and DEPTOR, which regulates protein translation and energy metabolism [5,6]. The latter (mTORC2) is a complex including mTOR, Rictor, Sin1, as well as Protor and DEPTOR, which regulates the kinase activity of Akt [5,6]. Inhibitors of mTORC1 (rapamycin and its analogs, or “rapalogues”) have shown activities in RCC patients. Two of such agents, temsirolimus (CCI-779) and everolimus (RAD001) were approved by USA FDA for treatment of advanced or recurrent RCC [7,8]. However, responses to these mTORC1 inhibitors are infrequent and typically short lived, and after treatment all patients eventually develop progressive disease [9,10].

Rapalogues only partially block mTORC1 and do not inhibit mTORC2. Furthermore, mTORC1 inhibition could lead to the activation of phosphatidylinositol 3-kinase (PI3K)/AKT that counteract the anticancer efficacy of rapalogues. To overcome these limitations, ATP-competitive inhibitors of mTOR have been developed [11]. These inhibitors target the ATP site of mTOR kinase domain, thereby blocking mTORC1 and mTORC2 simultaneously [11]. The most distinguishing advantage of these compounds (i.e. AZD-8055, AZD-2014, INK-128, and OSI-027), besides exerting a more efficient mTORC1 inhibition, would be the mTORC2 blockade leading to significant decrease of AKT Ser473 phosphorylation [11]. In this study, we found that AZD-2014, a novel mTOR ATP-competitive blocker [12], inhibited RCC cell growth both in vitro and in vivo with a much higher efficiency than rapalogues.

Part of the study also focused on the role of autophagy in AZD-2014’s activity against RCC cells. Autophagy is an adaptive process where cells clear the damaged proteins or organelles through lysosomal degradation, thus providing energy and nutrients to survive...
In cancer cells, autophagy activation is considered as a resistance factor, exerting pro-survival and anti-apoptosis activities [13,14]. Numerous studies have reported autophagy activation after anticancer therapies in different cancer cell lines. Accordingly, autophagy inhibition could sensitize multiple anticancer-targeted therapies [13,15]. We here observed autophagy activation by AZD-2014 in RCC cells, while inhibition of autophagy by pharmacologic or genetic means enhanced activity of AZD-2014.

Materials and methods

Reagents and chemicals

AZD-2014, rapamycin and everolimus (RAD001) were obtained from Selleck China (Shanghai, China). Chloroquine (Cq) and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The broad caspase inhibitor z-VAD-fmk and the caspase-8 specific inhibitor z-ITED-fmk were from Calbiochem (Darmstadt, Germany). Anti-light chain 3B (LC3B), Raptor, Rictor, Sin1, mTOR, Beclin-1, autophagy protein 5 (ATG-5), tubulin, hypoxia-inducible factor (HIF)-1α, HIF-2α and cyclin D1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for p-AKT (Ser-473), p-AKT (Thr-308), p-STAT3 (Ser-233/234), p-ERK1/2, p-ERK1/2 (Thr-202/Tyr-204), ERK1/2, p-S6K1 (Thr-389), S6K1, cleaved-caspase 3, cleaved-Poly (ADP-ribose) polymerase (PARP) and tubulin were obtained from Cell Signaling Technologies (Beverly, MA, USA). Antibodies for forhead box O 3α (FoxO3α) and p-FoxO3α (Thr-32) were purchased from Abcam (Cambridge, MA, USA).

Cells

Human RCC cell lines 786-0 and A498 were purchased from Shanghai Biological Institute (Shanghai, China). RCC cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). HK-2 cells, an immortalized proximal tubule epithelial cell line from adult human kidney [16], were cultured in DMEM/Ham's F12 (Life Technologies Ltd., Paisley, UK) supplemented with 10% FBS, 2mM glutamine (Life Technologies Ltd.). 20 mM HEPES buffer, 0.64 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma).

Methylthiazol tetrazolium (MTT) assay

Cell survival was assessed through MTT assay with recommended protocol (Roche Diagnostics). In brief, cells were collected and seeded in 96-well plates at a density of 5 × 10^4 cells/well in 200 μl of culture medium (containing 1% FBS). After treatment, MTT solution was added to each well for 2 h at 37 °C, cell viability was determined by measuring absorbance using a microplate spectrophotometer ( Molecular Devices, Sunnyvale, CA, USA). OD value was utilized as the indicator of cell survival in vitro.

Clonogenicity assay

Cells were seeded and allowed to attach overnight. Next day, cells were treated with indicated drug. Following the treatment, the cells were re-fed with drug containing medium every 2 days. After 10 days, the colonies were stained with crystal blue/violet and counted. The number of colonies in treatment group was normalized to that of untreated control group.

Annexin V assay of cell apoptosis

Cells were washed and incubated in 500 μl binding buffer, 5 μl annexin V-FITC and 5 μl of propidium iodide (PI) (Invitrogen, Karlsruhe, Germany) at room temperature for 15 min, which were then detected through fluorescence-activated cell sorting (FACS) with a Becton-Dickinson machine (San Jose, CA, USA). Annexin V positive cells were labeled as apoptotic cells, and its percentage was recorded as the indicator of apoptosis intensity.

Caspase-3 activity assay

The cytosolic proteins from approximately 1 × 10^6 cells were extracted in hypotonic cell lysis buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl2, 5 mM EDTA, 5 mM dithiothreitol, 0.05% phenylmethylsulfonyl fluoride. Ten micromolars of caspase-3 activity assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzoylacetonyl-DEVD-7-amido-4-(trifluoromethyl) coumarin as substrates (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-ethylcoumarin as substrates (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-ethylcoumarin was quantified, after 2 h of incubation at 37 °C, using a Fluoroskan system set to an excitation value of 355 nm and emission value of 525 nm.

Results

AZD-2014 inhibits RCC cell survival and growth, more efficiently than rapamycin and RAD001

We tested the potential role of AZD-2014 on RCC cell survival. MTT viability assay was performed, and results demonstrated that AZD-2014 dose-dependently inhibited survival of 786-0 cells and A498 cells (Fig. 1A and B), and its efficiency was significantly higher than same concentration of conventional mTORC1 inhibitors rapamycin or RAD001 (Fig. 1A and B). The IC-50s for AZD-2014, RAD001 and rapamycin were 52.25 ± 3.12 μM, 262.31 ± 18.21 μM, and 5.500 μM in 786-0 cells, and were 22.54 ± 2.12 μM, 234.25 ± 12.75 μM, and 6.500 μM in A498 cells. Results in Fig. 1C and D showed that the effect of AZD-2014 was also time-dependent. In both RCC cell lines, no significant viability decrease was achieved.
AZD-2014 inhibits RCC cell survival and growth, more efficiently than rapamycin and RAD001. 786-0 cells and A498 cells were treated with indicated concentrations of AZD-2014, rapamycin or RAD001 for 72 h (A and B), or stimulated with AZD-2014 (100 nM) for indicated hours (C and D), cell viability was tested by MTT assay (n = 5). 786-0 cells and A498 cells were cultured in culture medium (5% FBS) or conditional medium with AZD-2014 (50/100 nM), rapamycin (100 nM) or RAD001 (100 nM) for 10 days, the surviving colonies were manually counted (E and F) (n = 5). Experiments in this and the following figures were repeated three times, and similar results were obtained. *p < 0.05 vs. AZD-2014 (0 nM) group. **p < 0.05 vs. same concentration of AZD-2014 group.

AZD-2014 inhibits RCC cell survival and growth, more efficiently than rapamycin and RAD001. 786-0 cells and A498 cells were treated with indicated concentration of AZD-2014, rapamycin or RAD001 for 72 h, or stimulated with AZD-2014 (100 nM) for indicated hours. Cell viability was tested by MTT assay (n = 5). 786-0 cells and A498 cells were cultured in culture medium (5% FBS) or conditional medium with AZD-2014 (50/100 nM), rapamycin (100 nM) or RAD001 (100 nM) for 10 days, the surviving colonies were manually counted (E and F) (n = 5). Experiments in this and the following figures were repeated three times, and similar results were obtained. *p < 0.05 vs. AZD-2014 (0 nM) group. **p < 0.05 vs. same concentration of AZD-2014 group.

AZD-2014 induces RCC cell apoptosis

The effect of AZD-2014 on RCC cell apoptosis was tested next. Three independent assays including Western blots detecting apoptosis related proteins, FACS labeling Annexin V positive cells as well as caspase-3 activity assay were performed. Treatment time and AZD-2014 concentration were chosen based on pre-experimental results and published literatures (same for all experiments). Results showed that cytotoxic AZD-2014 (50–100 nM) induced caspase-3 and PARP cleavage in 786-0 cells (Fig. 2A); the percentage of Annexin V positive cells increased after AZD-2014 administration (Fig. 2B and C). The caspase-3 activity was also increased in AZD-2014-treated cells (Fig. 2D). Similar results were also seen in A498 cells (data not shown). These results confirmed apoptosis activation by AZD-2014 in RCC cells. Notably, high concentration of AZD-2014 (100–250 nM) only induced minimal apoptosis in HK-2 cells, which is an immortalized proximal tubule epithelial cell line from normal adult human kidney (Fig. 2E). Further, HK-2 cell viability after AZD-2014 (100–250 nM, 72 h) treatment was only slightly decreased (less than 10%) (Fig. 2F). These results suggest the specific cytotoxicity of AZD-2014 against RCC cells, but not in non-cancerous cells.

AZD-2014 disrupts mTORC1/2 assembly and activation, thus downregulating mTOR-regulated genes in RCC cells

AZD-2014 is recognized as mTORC1/2 dual inhibitor, we thus tested its role on mTORC1/2 assembly and activation in RCC cells. Western blot results showed that AZD-2014 (100 nM) almost blocked phosphorylation of S6K1, S6 and AKT (Ser473) in 786-0 cells and A498 cells (Fig. 3A, upper panel), indicating mTORC1 and mTORC2 dual suppression by AZD-2014. The effect of AZD-2014 on phosphorylation of AKT (Thr308), GSK3α and FoxO1a/a/3a was tested, and results demonstrated that AZD-2014 inhibited FoxO1a/a/3a phosphorylation (Fig. 3A, lower panel), but showed negligible or minor effect on phosphorylation of AKT (Thr308) and GSK3α in RCC cells (Fig. 3A). These results are not unexpected, as FoxO1a/a, similar to AKT Ser473, are downstream effectors of mTORC2 (“PDK2”) [18], while AKT-308 and GSK3α are downstream signals of PD1K1 [19]. Note that ERK1/2 phosphorylation was not affected by AZD-2014 in RCC cells (Fig. 3A, upper panel). Co-IP results in Fig. 3B and C demonstrated that AZD-2014 disrupted the assembly of mTORC1 (mTOR-raptor association) and mTORC2 (mTOR-Rictor-Sin1 association) in both 786-0 cells and A498 cells, expressions of mTOR complex components were not affected by AZD-2014 (see “Input” in Fig. 3).
AZD-2014 induces RCC cell apoptosis. 786-0 cells were treated with indicated concentration of AZD-2014, after 24 h, expressions of cleaved-caspase-5 and caspase-3 activity assay (D, \*p < 0.05 vs. AZD-2014 (0 nM) group. *MW" stands for molecular weight (all figures). 

AZD-2014 activates pro-survival autophagy in RCC cells

One of the consequence of mTOR inhibition is autophagy induction (see Discussion). Western blot results showed that AZD-2014 activated autophagy in 786-0 cells, which was detected by Beclin-1 and ATG-5 upregulation, LC3B-I to LC3B-II conversation as well as p62 degradation (Fig. 4A). 3-Methyladenine (3-MA) and chloroquine (Cq), two autophagy inhibitors, significantly increased AZD-2014-induced viability decrease (Fig. 4B) and apoptosis (Fig. 4C) in 786-0 cells, suggesting that AZD-2014-stimulated autophagy is anti-apoptosis and pro-survival. Importantly, the caspase-8 inhibitor z-ITED-fmk and the broad caspase inhibitor z-VAD-fmk largely inhibited AZD-2014 plus 3-MA-induced cytotoxicity and apoptosis in 786-0 cells (Fig. 4D), and similar results were also seen in A498 cells (data not shown). Together, these results indicate that autophagy activation by AZD-2014 serves as a resistance factor, and autophagy inhibitors sensitize AZD-2014’s in vitro activity through facilitating cell apoptosis.

SiRNA knockdown of Beclin-1 or ATG-5 increases activity of AZD-2014 in vitro

To further confirm the role of autophagy in AZD-2014’s action, siRNA strategy was applied to knockdown key autophagy participants (Beclin-1 or ATG-5). As demonstrated in Fig. 5A, targeted siRNA showed high efficiency and significantly downregulated Beclin-1 or ATG-5 expression in 786-0 cells. Similar to the inhibitor results, AZD-2014-induced cytotoxicity and apoptosis were largely enhanced by RNAi knockdown of Beclin-1 or ATG-5 (Fig. 5B and C), further supporting that autophagy inhibition, either through genetic or pharmacological means, could sensitize the activity of AZD-2014. Beclin-1 knockdown mediated AZD-2014 sensitization was again inhibited by the two caspase inhibitors (Fig. 5D). Together, these results show that in-activation of autophagy by genetic methods facilitates AZD-2014-induced in vitro activity in cultured RCC cells.

AZD-2014 shows higher efficiency than RAD001 in inhibiting RCC cell growth in vivo, and its activity could be further enhanced by the autophagy inhibitor 3-MA

To evaluate the in vivo activity of AZD-2014, nude/beige mice bearing 786-0 xenografts were treated daily with vehicle, RAD001, or AZD-2014 for 15 consecutive days. Xenograft assay results in Fig. 6A demonstrated that AZD-2014 administration resulted in significant growth arrest, whereas RAD001 had only a modest effect on tumor growth. The AZD-2014-treated group had the lowest tumor volume in 786-0 xenograft models at day 25 (985.9 ± 129.8 mm³), which was significantly smaller than the RAD001 group (1532.0 ± 239.6 mm³) or the vehicle-treated group (2029.2 ± 200.5 mm³) (Fig. 6A). There was a significant difference in the average tumor weight among the AZD-2014 group and RAD001 group on day 25 (Fig. 6B). The in vivo activity of AZD-2014 against 786-0 xenografts was further enhanced by 3-MA co-administration, resulting in even smaller tumor volume and lesser tumor weight (Fig. 6A and B). Note that the mice body weight was not significantly affected by above regimens, indicating the relative safety of the treatments (Fig. 6C).

To evaluate the in vivo effects of AZD-2014 and RAD001 on intracellular signalings, two mice per treatment group were sacrificed
after 5 days of treatment, and tumors were excised for analysis. Results demonstrated that although both AZD-2014 and RAD001 suppressed S6 phosphorylation (mTORC1 indicator) in tumor tissues, only AZD-2014 also inhibited AKT Ser473 phosphorylation (mTORC2 indicator) (Fig. 6D). Further, AZD-2014 administration resulted in a significant reduction of HIF-1α/HIF-2α expressions, while RAD001 had a softer effect (Fig. 6D). It should be noted that although there are many reports showing ERK activation by rapalogues [28,29], the role of these mTORC1 inhibitors on ERK activation has been inconsistent within different cancer cell lines [30–32]. Here we found that ERK1/2 phosphorylation was not affected by either RAD001 or AZD-2014 treatment (Fig. 6D). Our results are consistent with other studies showing that RAD001 had no significant impact on ERK activation in several other cancer cell lines [30–32]. Together, these results show that AZD-2014 is more efficient than RAD001 in inhibiting 786-0 xenografts, its activity could be further enhanced by the autophagy inhibitor 3-MA.

Discussion

Targeting mTOR is an effective approach in the treatment of advanced RCC. Hyperactivity of mTOR signaling is often seen in RCC, which correlates with aggressive behavior and poor prognosis [4,9]. MTORC1 and mTORC2 complexes are formed and regulated by different proteins and are also driven by multiple different compensatory feedback loops [11]. The cytotoxic activity and antiproliferative property of first generation of mTOR inhibitors (rapalogues) are limited [11]. Further, mTORC1 inhibition by rapalogues could result in a feedback activation of the PI3K-AKT [11]. Here we found that treatment of AZD-2014, a dual mTORC1/2
Fig. 4. AZD-2014 activates pro-survival autophagy in RCC cells. 786-0 cells were either left untreated (“C”), or treated with AZD-2014 (50 nM) for 12 or 24 h, expressions of indicated proteins were tested by Western blots (A), 786-0 cells were pre-added with 3-methyladenine (3-MA, 0.5 mM) or chloroquine (Cq, 10 μM) for 1 h, followed by AZD-2014 (50 nM) stimulation, cells were further cultured in drug-containing medium, cell viability was tested by MTT assay 72 h after AZD-2014 stimulation (B), and cell apoptosis was tested by Annexin V FACS assay 48 h after AZD-2014 stimulation (C). 786-0 cells were pre-added with 3-methyladenine (3-MA, 0.5 mM) or chloroquine (Cq, 10 μM) for 1 h, followed by AZD-2014 (50 nM) plus 3-methyladenine (3-MA, 0.5 mM)/AZD2014 (50 nM) stimulation, cells were further cultured in drug-containing medium, cell viability and apoptosis were analyzed similarly. Beclin-1, ATG-5, LC3B-II and p62 expressions were quantified. *p < 0.05.

Fig. 5. siRNA knockdown of Beclin-1 or ATG-5 increases activity of AZD-2014 in vitro. Expressions of Beclin-1, ATG-5 and tubulin (loading) in 786-0 cells transfected with scramble siRNA (“SC siRNA”), Beclin-1 siRNA or ATG-5 siRNA were shown (A). 786-0 cells transfected with above siRNA (SC siRNA, Beclin-1 siRNA or ATG-5 siRNA) were either left untreated (“C”), or treated with AZD-2014 (50 nM), cells were further cultured in drug-containing medium, cell viability was tested by MTT assay 72 h after AZD-2014 stimulation (B), and apoptosis (48 h after AZD-2014 stimulation) were tested by Annexin V FACS assay (C), respectively. 786-0 cells transfected with Beclin-1 siRNA were pre-treated with z-VAD-fmk (VAD, 50 μM) or z-ITED-fmk (ITED, 50 μM) for 1 h, followed by AZD-2014 (50 nM) stimulation, cells were cultured in drug-containing medium, cell viability and apoptosis were analyzed similarly (D). Beclin-1 and ATG-5 expressions were quantified. *p < 0.05.

Inhibitor, significantly inhibited RCC cell (786-0 and A498 cell lines) growth both in vivo and in vitro, and its efficiency is significantly higher than conventional mTORC1 inhibitors (RAD001 and rapamycin). Thus, the concurrent suppression of mTORC2 in addition to mTORC1 is likely to be a more effective strategy in the treatment of RCC than inhibition of mTORC1 alone.

A strong association exists between pVHL (von Hippel–Lindau protein) inactivation and RCC progression. pVHL is in the E3 ubiquitin ligase complex, responsible for the degradation of HIF-1α/2α [33]. Following pVHL inactivation, HIF-1α/2α accumulate and dimerize with HIF3α, resulting in the expression of vascular endothelial growth factor (VEGF) and many other pro-angiogenic factors [33]. Somatic mutations of VHL are seen in up to 50% of sporadic RCC [33]. Emerging evidences have shown that HIF-2α is far more important than HIF-1α in the pathogenesis of RCC. Indeed, HIF-2α silencing inhibits the ability of pVHL knockout RCC cells to form tumors in vivo [34]. Tumor suppression function of pVHL is abolished with overexpression of HIF-2α but not HIF-1α [35]. Meanwhile, it has been shown that translation of HIF-2α is mainly dependent upon the activity of mTORC2 [36]. We found that AZD-2014 markedly reduced the expression of HIF-2α and HIF-1α in vivo and in vitro, whereas RAD001 had almost no effect on HIF-2α, and modest effect on HIF-1α. These observations are consistent with a recent study showing that NVP-BEZ235, a dual PI3K/mTOR inhibitor, significantly inhibited HIF-2α expression, while rapamycin had no such effect [37]. Thus, we suggest that dual block of mTORC1 and mTORC2 is far more efficient than mTORC1 block in decreasing HIF-1α/2α expressions, which play key roles in RCC progression.

mTORC1 is a key regulator of autophagy. Activated mTORC1 promotes growth and inhibits autophagy by interacting with unc-51-like kinase 1 (ULK1) [38] and autophagy-related protein 13 (ATG13) [39], thus inhibiting ULK1 complex. MTORC1 inhibition thus promotes dissociation of mTOR from the ULK1 complex. This frees ULK1 to activate FIP200, which mediates autophagosome formation to initiate autophagy [39]. Recent studies have also characterized the possible role of mTORC2 in autophagy, and showed that mTORC2 similarly represses autophagy through regulating AKT/FoxO3a signaling pathway [40,41]. For example, Mammucari et al. showed that mTORC2 suppression through Rictor knockdown induced FoxO3a like kinase 1 (ULK1) [38] and autophagy-related protein 13 (ATG13) [39], thus inhibiting ULK1 complex. MTORC1 inhibition thus promotes dissociation of mTOR from the ULK1 complex. This frees ULK1 to activate FIP200, which mediates autophagosome formation to initiate autophagy [39]. Recent studies have also characterized the possible role of mTORC2 in autophagy, and showed that mTORC2 similarly represses autophagy through regulating AKT/FoxO3a signaling pathway [40,41]. For example, Mammucari et al. showed that mTORC2 suppression through Rictor knockdown induced FoxO3a nuclear translocation, thus allowing formation of autophagic vesicles [40]. In breast cancer cells, mTORC2/AKT/FoxO3a inhibition activated autophagy [41]. These results indicate that mTORC2 activation also participate in autophagy suppression [40,41]. In this study, we found that both mTORC1 (S6K1-S6 phosphorylation) and mTORC2
(AKT-Ser 473-FoxO3a phosphorylation) activations were blocked by AZD-2014, which could explain a significant autophagy activation in AZD-2014-treated cells.

One reason why this study was performed is that many findings showed that autophagy inhibition given in combination with anti-cancer agents could promote chemosensitization [13,15]. Here we discovered that autophagy blockage utilizing pharmacological or genetic approaches could significantly enhance AZD-2014 activity both in vitro and in vivo. The lysosomotropic drug chloroquine (Cq) disrupts autophagic protein degradation through increasing intra-lysosomal pH. 3-Methyladenine (3-MA) inhibits LC3B-I to LC3B-II conversion, thus disrupting autophagosome formation. Both drugs significantly increased AZD-2014-induced apoptosis and growth inhibition in vitro. 3-MA also sensitized AZD-2014-mediated in vivo activity against RCC cells. These results suggest that autophagy inhibitors may be developed as AZD-2014 adjuvant. Beclin-1 is required for the initiation of the autophagosome formation in autophagy.

Conclusions

Targeting mTOR with rapamycin and rapalogues has been a successful strategy in RCC. So far, several targeted agents (i.e. RAD001 and CCI-779) have been approved for various uses in advanced RCC patients. However, response rates and increased length of progression-free survival (≥6 months) for these agents are far from satisfactory. Our results show that AZD-2014, the ATP-competitive inhibitor of mTOR, inhibits RCC cell growth in vitro and in vivo more effectively than RAD001. Furthermore, its inhibitory activity on ATG-5 is an E3 ubiquitin ligase which is necessary for autophagy due to its role in autophagosome elongation [42]. We found that both proteins were upregulated by AZD-2014, and RNAi silencing of either protein sensitized AZD-2014-mediated autophagy exerts a pro-survival role, and autophagy inhibition could sensitize AZD-2014’s activity.
HIF-1α/2α expressions and AKT-mTOR activation is also superior to RAD001. Further, AZD-2014 activated protective autophagy in RCC cells, autophagy inhibition dramatically increased AZD-2014’s activity in vitro and in vivo. These results provide the rationale for the clinical assessment of ATP-competitive mTOR inhibitors (i.e. AZD-2014) in advanced RCC patients, and development of autophagy inhibitors as the adjuvants.

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Conflict of interest

There are no conflicts of interest.

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