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Simultaneous inhibition of mTORC1 and mTORC2 by mTOR kinase inhibitor AZD8055 induces autophagy and cell death in cancer cells

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mTOR is a major biological switch, coordinating an adequate response to changes in energy uptake (amino acids, glucose), growth signals (hormones, growth factors) and environmental stress. mTOR kinase is highly conserved through evolution from yeast to man and in both cases, controls autophagy and cellular translation in response to nutrient stress. mTOR kinase is the catalytic component of two distinct multiprotein complexes called mTORC1 and mTORC2. In addition to mTOR, mTORC1 contains Raptor, mLST8 and PRAS40. mTORC2 contains mTOR, Rictor, mSIN1 and Protor-1. mTORC1 activates p70S6K, which in turn phosphorylates the ribosomal protein S6 and 4E-BP1, both involved in protein translation. mTORC2 activates AKT directly by phosphorylating Serine 473. pAKT(S473) phosphorylates TSC2 (tuberin) and inactivates it, preventing its association with TSC1 (hamartin) and the inhibition of Rheb, an activator of mTOR. pAKT also phosphorylates PRAS40, releasing it from the mTORC1 complex, increasing its kinase activity. Finally, AKT regulates FOXO3 phosphorylation, sequestering it in the cytosol in an inactive state.

mTORC1 is a negative regulator of autophagy, directly by phosphorylating ULK1 and preventing ULK1-Atg13-FIP200 complex formation, and indirectly by phosphorylating S6K and 4E-BP1. In stress conditions (low energy, low amino acids, hypoxia or ER stress), mTORC1 activation is rapidly switched off by multiple upstream mechanisms: Low glucose or a high AMP/ATP ratio activates the energy sensor LKB1-AMPK pathway which decreases mTORC1 activity, both indirectly via TSC2 and directly via phosphorylation of raptor; hypoxia and ER stress, by activating REDD1 also reduce mTORC1 activity; decrease in amino acids affects MAP4K3 and Rag GTPases, acting upstream of mTORC1. More recently, reports on the involvement of mTORC2 in autophagy have started to emerge. The mTORC2 protein rictor regulates autophagy, independently of mTORC1 in muscle cells in fasting conditions. A subsequent decrease in AKT activity allows FoxO transcription factors to enter the nucleus and transcriptionally regulate some key autophagy genes such as *LC3B*, *Gabarap*, *ULK2* and beclin 1. In these conditions, rapamycin, which principally inhibits mTORC1, is unable to induce autophagy. The regulation of autophagy processes by both mTORC complexes suggests that inhibition of mTOR kinase activity, by having an impact on both mTORC1 and mTORC2, may have a greater effect on autophagy, and possibly cell death, compared to rapamycin.

Our study describes AZD8055, a first-in-class orally available, potent and selective mTOR kinase inhibitor. In the H838 LKB1-deficient lung cancer cell line, AZD8055 induces the formation of punctate acidic vesicles in the cytoplasm detected by acridine orange indicative of an increase in autophagy (**Fig. 1**). Furthermore, immunostaining with antibodies to light chain 3 (LC3) revealed a concentration-dependent increase in the

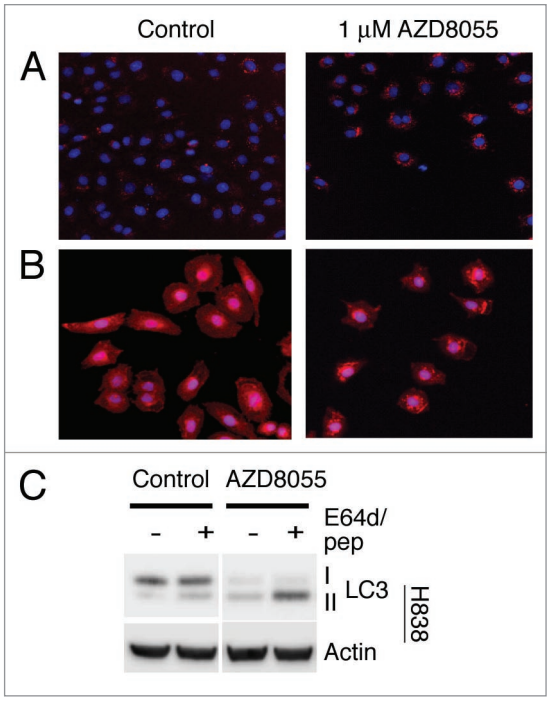

Figure 1. Figure 1: 1 μmol/l AZD8055 induces autophagy in H838 cells. Acridine staining (A) and LC3 staining (B) determined by immunofluorescence in H838 cells exposed to vehicle or AZD8055. C, expression of LC3 (LC3-I and LC3-II) determined by immunoblotting in cell extracts from H838 cells exposed to AZD8055 for 48h in presence or absence of E64d and pepstatin

punctate staining pattern, consistent with LC3 being localized to these acidic vesicles. Finally, LC3-I is normally converted into LC3-II (LC3-I covalently bound to phosphatidylethanolamine) during autophagosome formation, and is subsequently degraded by protease cleavage during autolysosome maturation. To gain insight into the amount of LC3-II being generated, the cells were preincubated with the lysosomal inhibitors E64d and leupeptin, thus inhibiting protease-induced degradation of LC3-II. AZD8055 increases LC3-II levels and this increase is more pronounced in the presence of E64d and leupeptin, consistent with an increase in autophagosome formation. Additionally, while rapamycin and analogues induce in most cell lines a partial inhibition of cell growth, AZD8055 induces a profound growth inhibition, and in some cell lines, cell death.

We confirmed that these effects are driven by both mTORC1 and mTORC2 inhibition. AZD8055 decreases S6K phosphorylation. It also decreases 4EBP1 phosphorylation, not only on Ser65/70 (rapamycin-sensitive) but also on Thr37/46 (insensitive to rapamycin). This leads to a greater inhibition of cap-dependent protein translation compared to rapamycin. AZD8055 also potently inhibits phosphorylation of AKT and its downstream substrates PRAS40, GSK3β and TSC2. A decrease in phosphorylation of FoxO1/3a is also observed, leading to the relocalization of FoxO proteins in the nucleus.

Our data suggest that a complete inhibition of mTORC1 coupled with mTORC2 inhibition is likely to account for the greater inhibition of cell proliferation and greater induction of autophagy observed with AZD8055 compared to rapamycin.

In cancer, the existing literature reports that autophagy is induced in response to treatment with several targeted therapies and cytotoxic agents. It is suggested that autophagy may promote cancer cell survival and resistance to therapies. mTOR plays a crucial role in cancer cells, which tread a fine line between growth and death in ever-changing environmental conditions. The fact that LKB1 and TSC2 are mutated or lost in certain cancers may constitute a growth advantage during tumorigenesis in overcoming prolonged autophagy and induction of senescence. Feedback loops are also essential for autophagy: Induction of autophagy and inhibition of protein synthesis should result in accumulation of amino acids, restoring to some degree mTORC1 activation. It is possible that AZD8055, by maintaining low mTORC1 activity during prolonged autophagy, can lead to cell death. The deficiency of LKB1 in H838 cells may have also contributed to the induction of prolonged autophagy. However, this is likely to be more complex as AZD8055 induces autophagy in some but not all cell lines studied. Similarly, cell death is observed in conjunction with autophagy but sometimes, in the absence of autophagy. Senescence was not evaluated in these studies but it is also linked to autophagy and mTOR inhibition.

Both laboratory-based studies on autophagy and clinical trials have combined targeted therapies with lysosomal disruptors such as chloroquine, suggesting that autophagy is detrimental to antitumor activity. It is noteworthy that AZD8055, by inhibiting mTORC1 and mTORC2, induces autophagy and cell death. Unraveling the interplay between these two mechanisms may clarify in which tumors induction or inhibition of autophagy might be most beneficial. It might also provide new therapeutic targets.