Co-treatment of LY294002 or MK-2206 with AZD5363 Attenuates AZD5363-induced Increase in the Level of Phosphorylated AKT

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Abstract. Clinical trials are in progress on AZD5363, an inhibitor of protein kinase B (AKT), to assess its effects on the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. Cells treated with AKT inhibitors have been reported to activate alternative pathways in order to escape growth inhibition. AZD5363-sensitized Hs578T breast cancer cells displayed reduced levels of phosphorylated glycogen synthase kinase 3 beta (pGSK3β). Interestingly, in AZD5363-treated cells, the level of phosphorylated (activated) AKT (pAKT) increased. Since pAKT positively correlates with cancer growth and survival, we aimed to identify conditions that could reduce AZD5363-induction of pAKT. We examined whether AZD5363 induction of pAKT could be reduced by co-treatment with inhibitors of the PI3K/AKT/mTOR pathway. We observed that co-treatment of LY294002 or MK-2206 with AZD5363 reduced the level of pAKT. Since MK-2206 is clinically used, we propose that co-treatment using MK-2206 with AZD5363 would prove beneficial in blocking the AZD5363-induced pAKT signaling pathway. Our findings contribute to the development of AZD5363-based sensitization therapies for patients with cancer.

Materials and Methods

Reagents. AZD5363, MK-2206, wortmannin, perifosine, rapamycin, everolimus, and temsirolimus were supplied by Selleckchem (Houston, TX, USA). LY294002 was supplied by Calbiochem (Bellerica, MA, USA). Vinblastine was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Antibodies. Antibodies against AKT, pAKT, phosphorylated glycogen synthase kinase 3 beta (pGSK3β), phosphorylated...
ribosomal protein S6 kinase beta-1 (p70S6K), phosphorylated pyruvate dehydrogenase lipoamide kinase isozyme 1 (pPDK1), phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (p4EBP1), proliferating cell nuclear antigen (PCNA), and cleaved poly ADP ribose polymerase (C-PARP) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), survivin, and phosphorylated retinoblastoma protein (pRb) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against pmTOR were from Abcam (Cambridge, UK). Antibody against cyclin D1 was from Biosource (Camarillo, CA, USA).

Cell culture. Hs578T breast cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea), and were used previously (17-19). Human oral squamous carcinoma cell line, KB and its multidrug-resistant subline, KBV20C, were obtained from Dr. Yong Kee Kim, and were previously described (20). All cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Daegu, South Korea), and were used previously described trichloroacetic acid (TCA) method (17, 18). We observed that LY294002 is an established PI3K/AKT pathway inhibitor and relatively lower concentrations of AZD5363 could reduce AZD5363-induced cellular growth or survival signaling. We investigated conditions that would attenuate AZD5363 induction of pAKT. Since AZD5363 targets the AKT signaling pathway, we evaluated whether AZD5363 down-regulated expression of AKT pathway-related proteins. As shown in Figure 1B, AZD5363 reduced levels of AKT, pGSK3β, and pRb protein. Interestingly, the level of pAKT markedly increased in response to AZD5363 treatment. This suggests that the phosphorylation state of AKT had no effect on growth inhibition by AZD5363. It seems that AZD5363 affects AKT signaling pathway increasing the pAKT level, thereby inducing resistance to drug. Since an increased pAKT level could increase cellular growth signaling and survival to produce undesirable drug-resistant cell phenotypes, we investigated conditions that would attenuate AZD5363 induction of pAKT.

Co-treatment with LY294002 reduced AZD5363 induction of pAKT. We tested whether co-treatment with PI3K/AKT/mTOR inhibitors attenuated pAKT induction by AZD5363. LY294002 is an established PI3K/AKT pathway inhibitor (17, 18). We observed that LY294002 inhibited the growth of and reduced the pGSK3β level in Hs578T cells, similarly to that induced by AZD5363 treatment (Figure 1C and Figure 2B). This suggests that PI3K/AKT pathway-targeting inhibitor could sensitize Hs578T cells.

Next, we tested whether AZD5363 induction of pAKT was attenuated by co-treatment with LY294002. Co-treatment of LY294002 with a high concentration of AZD5363 reduced the pAKT level (Figure 1C), indicating that PI3K/AKT inhibitor ameliorates the effect of AZD5363 on pAKT. This also suggests that co-treatment of LY294002 could reduce AZD5363-induced cellular growth or survival signaling. We analyzed this effect in detail. Two different concentrations of LY294002 and relatively lower concentrations of AZD5363 were used for an increased incubation period (up to 2 days) to determine whether prolonged co-treatment with LY294002 and AZD5363 reduced the pAKT level. As displayed in Figure 1D, AZD5363-induced increase in pAKT levels even at a lower concentration (10 μM) and this increase was effectively reduced by both concentrations of LY294002 (10 μM and 20 μM). AZD5363 induction of pAKT was reduced by LY294002 in a dose-dependent manner (Figure 1D). Collectively, our results demonstrate that co-treatment of LY294002 reduces AZD5363 induction of pAKT.

Results

AZD5363-treated cancer cells display reduced level of pGSK3β, but increased level of pAKT. AKT-targeting drugs specifically sensitize cancer cells since they depend on AKT signaling for growth or survival. Firstly, in order to identify an AZD5363-susceptible cancer cell line, we tested growth inhibition of three cancer cell lines, KB (vinblastine-sensitive cell line), KBV20C (vinblastine-resistant cell line), and Hs578T cells. As shown in Figure 1A and 2A, Hs578T cells were sensitive to inhibition by AZD5363, whereas both drug-sensitive KB and drug-resistant KBV20C cell lines were not at similar concentrations. Furthermore, we analyzed mechanism of action of AZD5363 in the Hs578T breast cancer cell line.
in reducing cellular growth and pGSK3β level in Hs578T cells (Figure 3A and Figure 2C). We observed that only AZD5363 treatment increased pAKT whereas LY294002, MK-2206, wortmannin, and perifosine alone did not affect the pAKT level (Figure 1C and D, and 3A).

Next, we tested whether AZD5363 induction of pAKT was attenuated by co-treatment with these inhibitors. As shown in Figure 3B-D, only MK-2206 co-treatment with AZD5363 reduced AZD5363 induction of pAKT. The dose-dependent pAKT reduction by MK-2206 was observed at 1 day and sustained for 2 days. However, wortmannin and perifosine treatment for both 1 and 2 days did not affect the pAKT level (Figure 3B-D). LY294002-induced reduction of pAKT was more effective than that of MK-2206 (Figure 3E).

Collectively, our results indicate that co-treatment of LY294002 or MK-2206 with AZD5363 reduces AZD5363 induction of pAKT. Considering that MK-2206 is now in clinical use, the harmful effects of AZD5363-induced long-term activation of pAKT might be ameliorated by the beneficial effect of its co-treatment with MK-2206.

Our next step was to test whether the co-treatment of LY294002 or MK-2206 with AZD5363 influenced the activation or levels of the signaling proteins that function upstream and downstream of the PI3K/AKT/mTOR pathway (17, 18, 25, 26). We analyzed phosphorylated forms of the major proteins, mTOR, P70S6K, 4EBP1, and...
Figure 4A indicates that the activation states of all the proteins were similar after single AZD5363 treatment and after co-treatment with LY294002 or MK-2206 with AZD5363. Since the PI3K/AKT/mTOR pathway regulates cellular proliferation and survival (17, 18, 25, 26), we also checked for any alteration in the levels of cell cycle- and proliferation-related proteins (pRb, cyclin D1, PCNA, and survivin). As displayed in Figure 4A, all protein levels were generally similar after AZD5363 treatment alone and co-treatment with LY294002 or MK-2206. These results suggest that AZD5363 treatment alone was sufficient to reduce the levels of cell cycle- and proliferation-related proteins.

We hypothesized that co-treatment of AZD5363 and LY294002 or MK-2206 increases apoptosis of cancer cells, by reducing pAKT levels. However, we did not observe any increase in C-PARP level under the co-treatment conditions compared with use of AZD5363 alone (Figure 4B and C). Microscopic observations did not reveal any decrease in cellular proliferation for co-treatment with AZD5363 and LY294002 or MK-2206 (Figure 5A and B). This suggests that the reduction of pAKT levels by co-treatment with LY294002 or MK-2206 is too small for growth inhibition to be detected with current assay systems such C-PARP production or microscopic observation with 1- or 2-day duration.

Co-treatment with mTOR inhibitor does not reduce AZD5363 induction of pAKT. Next, we tested whether co-treatment with mTOR inhibitor would reduce AZD5363 induction of pAKT.
Three inhibitors that are in clinical use, namely rapamycin, everolimus, and temsirolimus (27-29), were selected and investigated to identify their potential for co-treatment with AZD5363. We observed that rapamycin, everolimus, and temsirolimus reduce growth and pGSK3β level in Hs578T cells relative to the control (Figure 5C and Figure 6A), suggesting that mTOR pathway-targeting inhibitors can sensitize Hs578T cells with reduction of AKT pathway. AZD5363 alone increased the pAKT level, while mTOR inhibitors did not influence the pAKT level (Figure 6A). Next, we evaluated whether co-treatment with these inhibitors attenuated pAKT induction by AZD5363. As indicated in Figure 6A, none of the co-treatments with the inhibitors reduced AZD5363 induction of pAKT. In addition, the PI3K/AKT/mTOR-related protein levels were generally similar after AZD5363 treatment alone and co-treatment with rapamycin, everolimus, or temsirolimus (Figure 6B). The C-PARP level did not increase under co-treatment conditions (Figure 6C), and microscopic examinations did not show any decrease in proliferation (Figure 5C).

Collectively, our results indicate that co-treatment of mTOR inhibitors with AZD5363 did not reduce AZD5363 induction of pAKT. This suggests that clinically used MK-2206 proves to be the most potential candidate for co-treatment with AZD5363 to minimize AZD5363 induction of pAKT.

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**Figure 3.** Co-treatment with MK-2206 reduces AZD5363 induction of phosphorylated protein kinase B (pAKT). Hs578T cell extracts were collected at 24 h (A), at 24 h or 48 h (B-D), or at 48 h (E) after treatment with agents singly or in combination, then used for western blot analyses. A: Treatment with AZD-20: 20 μM AZD5363; MK-0.5: 0.5 μM MK-2206; Per-4: 4 μM perifosine; Wor-1: 1 μM wortmannin; AZD+MK-0.5: 20 μM AZD5363 with 0.5 μM MK-2206; AZD+Per-4: 20 μM AZD5363 with 4 μM perifosine; AZD+Wor-1: 20 μM AZD5363 with 1 μM wortmannin; or 0.1% dimethylsulfoxide (Con). B-D: Treatment with AZD-10: 10 μM AZD5363; MK-0.2: 0.2 μM MK-2206; MK-0.5: Per-2/Per-4: 2/4 μM perifosine; Wor-1/Wor-2: 1/2 μM wortmannin; AZD+MK-0.2: 10 μM AZD5363 with 0.2 μM MK-2206; AZD+MK-0.5: AZD+Per-2+A/AZD+Per-4: 10/20 μM AZD5363 with 2/4 μM perifosine; AZD+Wor-1/AZD+Wor-2: 10/20 μM AZD5363 with 1/2 μM wortmannin; and 0.1% dimethylsulfoxide (Con). E: Treated with AZD-10; LY-20: 20 μM LY294002; MK-0.5: AZD+LY 10 μM AZD5363 with 20 μM LY294002; AZD+MK: 10 μM AZD5363 with 0.5 μM MK-2206; or 0.1% dimethylsulfoxide (Con). pGSK3β: Phosphorylated glycogen synthase kinase 3 beta, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
Discussion

AZD5363 is a novel promising drug that inhibits AKT isoforms (1-4). Interestingly, we found that AZD5363 increases the pAKT level in cancer cells. Of the eight known inhibitors of PI3K/AKT/mTOR pathway: AZD5363, LY294002, MK-2206, wortmannin, perifosine, rapamycin, everolimus, and temsirolimus, only AZD5363 increased the pAKT level. However, all of them reduced the pGSK3β level. Previous studies reported that AKT activation increases cancer cell survival and resistance to apoptosis induced by drugs (17, 18, 30). We hypothesized that AZD5363 induction of pAKT positively correlates with alternative survival pathways of cancer cells. Therefore, we attempted to identify mechanisms that reduce AZD5363 induction of pAKT in cancer cells in order to enhance the clinical use of AZD5363. We evaluated eight established inhibitors of the PI3K/AKT/mTOR pathway. The proliferation of Hs578T breast cancer cell was reduced by all eight inhibitors with reduction of PI3K/AKT/mTOR pathway. We assumed that growth inhibition induced by PI3K/AKT/mTOR targeting inhibitors is relatively similar in Hs578T breast cancer cells.

The prominent observation of this study is that AZD5363-induced increase in pAKT level was down-regulated by co-treatment with LY294002 or MK-2206. In particular, the role of MK-2206 is crucial, since it has been deemed safe and is clinically used to treat human patients (23). Patients
harboring a resistant phenotype and with an increased pAKT level from AZD5363 therapy might be effectively treated with MK-2206 co-treatment. We observed that LY294002 also effectively inhibited AZD5363 induction of pAKT. However, LY294002 is only an experimental inhibitor and not used in the clinical setting. LY294002 and its structural derivatives could potentially be used to study mechanisms of AZD5363 resistance induced by pAKT. Therefore, further studies are warranted to understand the mechanism(s) by which MK-2206 or LY294002 reduce pAKT expression. In future studies, we propose to measure dephosphorylation of AKT by PTEN or phosphorylation of AKT by PI3K, or unidentified kinases. The results of this might be applied for AZD5363-resistant cancer cells, which are the result of increased activation by pAKT.

We classified the eight inhibitors of PI3K/AKT/mTOR pathway based on molecular signatures. AZD5363 influences proteins similarly to LY294002 and mTOR inhibitors, whereas MK-2206 displayed a different pattern. This suggests that MK-2206 exhibits a different mechanism of cellular targeting from that of other PI3K/AKT/mTOR inhibitors. Since the PI3K/AKT/mTOR pathway regulates proliferation and survival signals (17, 18), we tested whether expression of cell cycle- and

Figure 5. Co-treatment of AZD5363 with phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) inhibitors does not reduce growth of AZD5363-treated cells. Hs578T cells were grown on 6-well plates and treated with agents singly or in combination. After 24 h, all cells were observed using an inverted microscope with a 5× objective lens. A and B: AZD-10: 10 μM AZD5363; MK-0.5: 0.5 μM MK-2206; LY-20: 20 μM LY294002; AZD+MK-0.5: 10 μM AZD5363 with 0.5 μM MK-2206; AZD+LY-20: 10 μM AZD5363 with 20 μM LY-294002; AZD-20: 20 μM AZD5363; AZD-20+MK-0.5: 20 μM AZD5363 with 0.5 μM MK-2206; AZD-20+LY-20: 20 μM AZD5363 with 20 μM LY-294002; or 0.1% dimethylsulfoxide (Con). C: Rap-10: 10 μM rapamycin; Eve-10: 10 μM everolimus; Tem-10: 10 μM temsirolimus; AZD+Rap: 20 μM AZD5363 with 10 μM rapamycin; AZD+Eve: 20 μM AZD5363 with 10 μM everolimus; AZD+Tem: 20 μM AZD5363 with 10 μM temsirolimus; AZD+MK: 20 μM AZD5363 with 0.5 μM MK-2206; AZD+LY: 20 μM AZD5363 with 20 μM LY-294002; or 0.1% dimethylsulfoxide (Con). After 24 h, all cells were observed using an inverted microscope with a 5× objective lens.
proliferation-related protein (cyclin D1, pRb, PCNA, and survivin) were altered. The protein levels after single treatment of AZD5363 were comparable to those after co-treatments of LY294002, MK-2206, or mTOR inhibitors. These results suggest that single treatment of AZD5363 was adequate to reduce the levels of growth signaling pathway proteins.

We hypothesized that the reduction in the level of pAKT by LY294002 or MK-2206 would result in sensitization to the growth-inhibitory effects of AZD5363; however, the growth of and apoptosis in cells after single AZD5363 treatment were comparable to that after co-treatments. The effect on pAKT level was minimal, and growth inhibition could not be detected using microscopic observations and analysis of C-PARP production. Further studies are warranted to analyze long-term effects.

In summary, our results contribute to the determination of the potential clinical use of MK-2206 for AZD5363-treated patients with cancer. The present study also enhances our understanding of AZD5363 mechanisms of action. Our findings provide insight into the development of AZD5363-based therapies for patients.

Conflicts of Interests

The Authors declare no conflicts of interests.
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References


