

Novel conjugates of endoperoxide and 4-anilinoquinazoline induce myeloma cell apoptosis by inhibiting the IGF1-R/AKT/mTOR signaling pathway

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SUMMARY 4-anilinoquinazoline-containing inhibitors of the epidermal growth factor receptor (EGFR) are widely used in non-small cell lung cancer patients with mutated EGFR, but they are less effective in multiple myeloma (MM), a fatal malignancy derived from plasma cells. The present study designed a series of novel compounds by conjugating a peroxide bridge to the 4-anilinoquinazoline pharmacophore. Further studies showed that these agents such as 4061 and 4065B displayed potent activity to induce MM cell apoptosis by upregulating pro-apoptotic p53 and Bax while downregulating pro-survival Bcl-2. The mechanistic analysis revealed that both 4061 and 4065B inhibited IGF1-R, AKT and mTOR activation in a concentration dependent manner but had no effects on the expression of their total proteins, suggesting the conjugates of endoperoxide and 4-anilinoquinazoline may exert its anti-myeloma activity by targeting the IGF1-R/AKT/mTOR pathway.

Keywords cell death, endoperoxide bridge, multiple myeloma, signaling transduction

1. Introduction

Multiple myeloma (MM) is a fatal hematological malignancy derived from plasma cells and accounts for approximately 2% of total cancer-associated death. The last few decades have witnessed the development of several lines of novel agents for the treatment of MM from immunomodulators, proteasomal inhibitors to monoclonal antibodies and CAR-T-based cell therapies. However, due to genetic heterogeneity and other factors, most patients quickly develop resistance and the general outcomes are not favorable (1). Until now, MM is still incurable and the five-year survival rate remains only 30 to 40 percent.

Insulin-like growth factor (IGF1) is a potent activator and promoter of the proliferation and survival of MM cells by activating the receptor tyrosine kinase (RTK) IGF-1 receptor (IGF1-R). It is reported that IGF1-R is hyperactivated in most MM cell lines and patient samples. As an RTK, IGF1-R activates phosphoinositide-3 kinase (PI3K) and subsequent

downstream signals, mainly the protein kinase B (AKT) and mammalian target of rapamycin (mTOR), therefore promoting MM cell proliferation and survival. Targeting the PI3K/AKT/mTOR signaling pathway has been regarded as a promising strategy for MM therapy and a number of specific inhibitors of this signaling pathway have been developed. Notably, PI3K is also activated by other RTKs, such as the epidermal growth factor receptor (EGFR), especially in solid cancers such as non-small cell lung cancers (NSCLCs). Inhibition of the EGFR pathway led to the discovery of specific inhibitors of EGFR (2), including gefitinib, erlotinib, afatinib and dacomitinib, all of which have been applied in the clinic for lung cancer patients. However, these inhibitors are less effective in liquid tumors such as MM (3). By comparing the chemical structure of these inhibitors, we found that all these inhibitors share the 4-anilinoquinazoline core scaffold (4). Therefore, it will be interesting to develop a second class of these inhibitors based on this scaffold for the treatment of MM. To develop such inhibitors, We took artemisinin

into consideration because it contains an endoperoxide pharmacophore and displays anti-MM activity (5). We therefore developed some novel conjugates of 4-anilinoquinazoline and endoperoxide pharmacophore (4). These novel compounds showed potency against NSCLC cell lines with L858R/T790M mutation in the EGFR (4). The present study found that 4061 and 4065B, two representative agents, displayed potent anti-MM activity by inhibiting the IGF1-R/AKT/mTOR signaling pathway.

2. Materials and Methods

2.1. Cell lines

MM cell lines OPM2, KMS11, OCI-My5 and JJN3 were generously provided by Dr. Aaron Schimmer from Ontario Cancer Center and the University of Toronto, Canada. MM cell lines RPMI-8226 and LP1 were purchased from American Type Culture Collection (Manassas, VA). All cell lines were maintained in Iscove's modified Dulbecco's medium. All media were supplemented with 10% fetal bovine serum, 100 µg/mL of penicillin, and 100 units/mL of streptomycin.

2.2. Gefitinib and novel conjugates of 4-anilinoquinazoline and endoperoxide

Gefitinib was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The novel conjugates of the 4-anilinoquinazoline and endoperoxide pharmacophore were synthesized and characterized by NMR as described previously (4).

2.3. Cell proliferation analysis

MM cells were treated with increasing concentrations of compounds of interest for 72 hrs before being subjected to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously (6).

2.4. Annexin V staining and flow cytometric analysis

Apoptosis was quantitatively assessed using Annexin V-FITC and propidium iodide (PI) staining (MultiSciences Biotech Co., Ltd., Hangzhou, China). After being treated with 4061 and 4065B at indicated concentrations for 24 hrs, cells were collected and rinsed with ice-cold PBS, re-suspended in 500 µL of 1 × binding buffer, and then labeled with 5 µL Annexin V-FITC and 10 µL PI for 10 min in the dark. The samples were mixed slightly before being analyzed on a flow cytometer (6).

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol[®] reagent (Sangon Biotech, Shanghai, China). Total RNA (2.5 µg) was reverse transcribed using a Superscript[™]-III kit (Invitrogen) according to the manufacturer's instruction. The polymerase chain reaction amplification was carried out in 25 µL of PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 20 pmol of each primer set, two units of Taq DNA polymerase (Transgen, Beijing, China), 0.2 mM dNTPs, and 2 µL cDNA. The Primers used were as follows: EGFR1, forward 5'-TTGCCGCAAAGTGTGTAACG-3' and reverse 5'-GTTGCACTTGCCACGCATT-3'; EGFR2, forward 5'-GCTCCTCCTCGCCCTCTT-3' and reverse 5'-GCCAGCTGGTTGTTCTTGTG-3'; FGFR3, forward 5'-CTGAAGAACGGCAGGGAGTT-3' and reverse 5'-CTGTGCGTCACTGTACACCT-3'; GAPDH, forward 5'-AATCCCATCACCATCTTCC-3' and reverse 5'-CATCACGCCACAGTTTCC-3'. Reaction cycling conditions were 3 min at 95°C, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, and 1 cycle at 72°C for 10 min. Products were analyzed on 2% agarose gels.

2.6. Analyses of the oncomine datasets

Several datasets from the Oncomine Database (<https://www.oncomine.org/>) were retrieved by searching genes of interest in association with MM and several solid cancers including NSCLC, breast cancer, liver cancer and prostate cancer tissues.

2.7. Western blotting

Cells (3×10^6) in 60-mm dishes were treated with 4061 and 4065B at indicated concentrations for 24 hrs before being collected for total cell lysates preparation in ice-cold lysis buffer. After clarifying using high speed centrifugation at 4°C, protein concentrations were determined with the BCA assay (Beyotime Institute of Biotechnology, Nantong, China). Equal amounts of proteins (30-40 µg) were fractionated using SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The blots were subjected to analysis against appropriate antibodies, including monoclonal PARP, Bcl-2, Bax, p53, IGF1-R, p-IGF1-R, p-AKT(S473), AKT, p-mTOR(S2448) and mTOR. All these antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-GAPDH antibody was purchased from Affinity Biosciences (Cincinnati, OH, USA).

2.8. Phosphorylation analysis

MM cell lines were stored overnight in Iscove's modified Dulbecco's medium containing 0.5% fetal bovine serum and low concentrations of 4061 and 4065B. One plate of cells was then treated with BENC-

511 for 2 hrs as a positive control. Cells were then stimulated with 100 ng/mL human recombinant insulin-like growth factor-1 (hrIGF-1, PeproTech, Rocky Hill, NJ) for 15 min before being lysed in Tris-buffered saline containing 1 mM sodium orthovanadate. After clarification, cell lysates were subjected to Western blotting analysis with anti-phospho-AKT (S473), anti-AKT, anti-phospho-mTOR (S2448) and anti-mTOR antibodies.

3. Results

3.1. 4061 and 4065B induce MM cell apoptosis

The EGFR inhibitors gefitinib, erlotinib, afatinib and dacomitinib share 4-anilinoquinazoline as the pharmacophore (Figure 1A), and artemisinin

and derivatives have endoperoxide moiety as the pharmacophore (7). To take advantage of both pharmacophores of these two classes of anti-cancer compounds, we designed and synthesized a series of novel agents by conjugating an endoperoxide bridge to the 4-anilinoquinazoline core scaffold (Figure 1B) (4). From a series of agents, two compounds, 4061 and 4065B (Figure 1B), had displayed improved activity to induce NSCLC cell death (4). To evaluate their anti-MM activity, MM cell lines RPMI-8226, LP1, and OPM2 were treated with 4061 and 4065B for 24 hrs, followed by Western blot assays to evaluate the cleaved level of PARP, a hallmark of apoptosis. As shown in Figure 1C, gefitinib, 4-anilinoquinazoline (YY) and endoperoxide (3022) failed to induce cleavage of PARP, in contrast, both 4061 and 4065B induced marked cleavage of PARP. To confirm this finding, MM

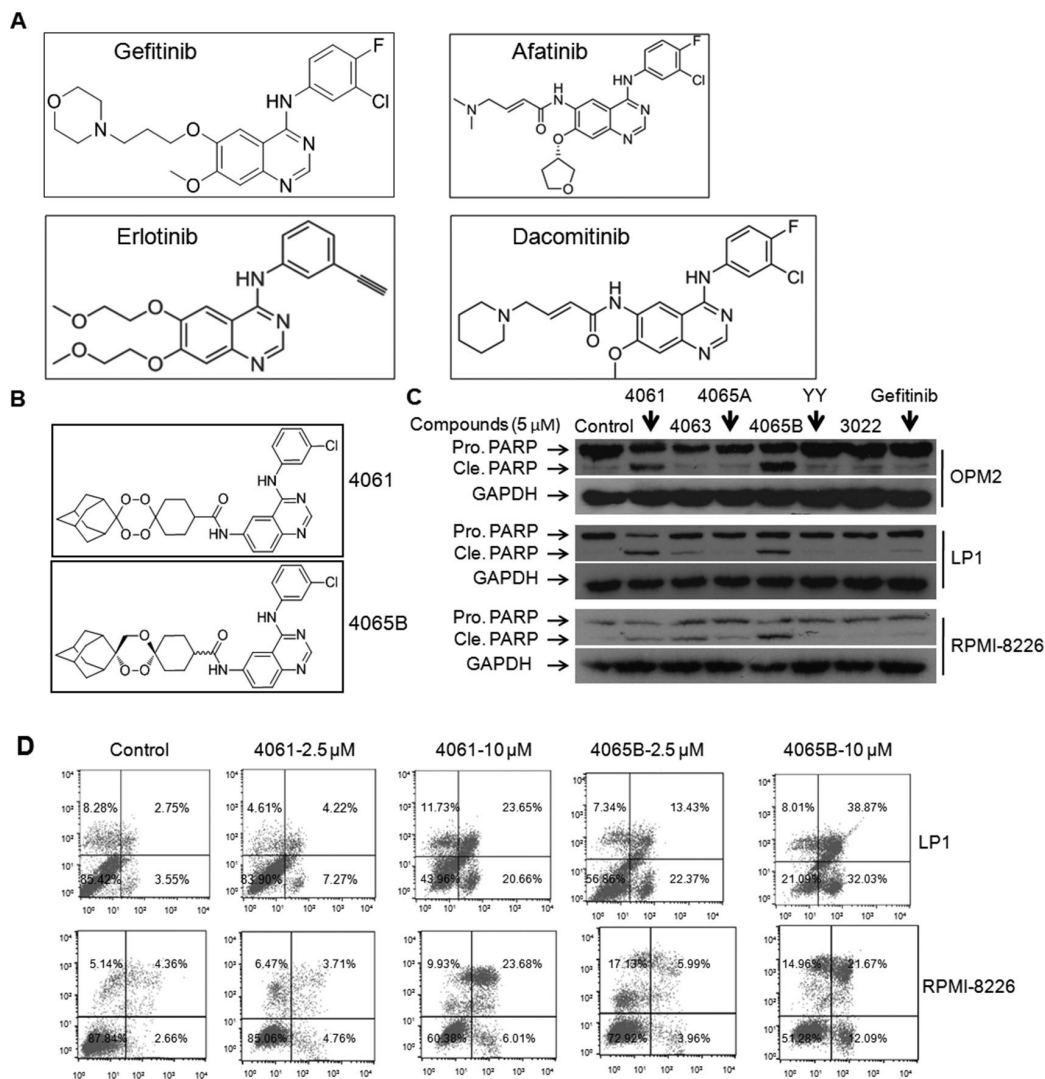


Figure 1. 4061 and 4065B induce MM cell apoptosis. (A) The chemical structure of Gefitinib and other EGFR inhibitors; (B) The chemical structure of 4061 and 4065B. (C) OPM2, LP1, and RPMI-8226 cells were treated with various compounds including gefitinib, 4061, 4065B and negative controls YY and 3022 for 24 hrs. Cell lysates were prepared and subjected to immunoblotting assay against apoptosis-associated proteins PARP. GAPDH was used as a loading control. (D) Apoptosis of LP1 and RPMI-8226 cells treated with various concentrations of 4061 and 4065B were determined by Annexin V-FITC and PI staining using flow cytometric analysis.

cells treated with these two compounds were stained with Annexin V and propidium iodide followed by flow cytometry analysis. When cells are undergoing apoptosis, phosphatidylserine inside the plasma membrane will turn over and appear on the cell surface and thus could be identified by Annexin V, therefore, Annexin V staining-positive cells are regarded as a gold criterion of apoptosis (6). Our study found that both 4061 and 4065B induced Annexin V positive cells in a concentration-dependent manner (Figure 1D). Therefore, 4061 and 4065B induce MM cell apoptosis.

3.2. 4061 and 4065B inhibit MM cell proliferation

To find out whether these two compounds also inhibit MM cell proliferation, a panel of MM cells were treated with 4061 and 4065B in increasing concentrations for 72 hrs, followed by the MTT assay. The results demonstrated that both 4061 and 4065B suppressed proliferation of all MM cell lines tested (Figure 2). Therefore, these conjugates of 4-anilinoquinazoline and endoperoxide suppress MM cell proliferation and induce MM cell apoptosis.

3.3. 4061 and 4065B upregulate pro-apoptotic signals

The above findings suggest both 4061 and 4065B induce MM cell apoptosis and suppress proliferation, to find out whether pro-apoptotic signals were activated, RPMI-8226 and LP1 cells were treated for 24 hrs followed by Western blotting assays to evaluate typical pro-apoptotic and pro-survival proteins. The results showed that these two analogs downregulated the protein level of Bcl-2, a typical pro-survival protein in cancer cells (Figure 3A and 3B), which was consistent with apoptosis and PRAP cleavage. In contrast, p53 and Bax, two typical pro-apoptotic proteins, were upregulated by both compounds (Figure 3A and 3B). Therefore, these results further demonstrated that these novel agents with conjugated endoperoxide bridge and the 4-anilinoquinazoline core scaffold display potent

activity in inducing MM cell apoptosis.

3.4. EGFR expression is dispensable for 4061 and 4065B to induce MM cell apoptosis

Gefitinib and derivatives are selective EGFR inhibitors mainly prescribed for NSCLC patients with mutated and overactivated EGFR (8), while it was ineffective in MM cells (3). Therefore, we wondered whether EGFR might not be expressed in MM cells. To this end, we first evaluated the expression profile of EGFR in various types of cancers, including NSCLC, breast cancer, colorectal cancer, lymphoma, melanoma and ovarian cancer. The result showed that EGFR was highly expressed in solid cancers but not in liquid cancers (Figure 4A). We next evaluated EGFR in two different MM datasets, the Zhan Myeloma dataset (9) and the Agnelli Myeloma 3 dataset (10), it turned out that EGFR is not in MM cells as expected (Figure 4B and 4C). To confirm these findings, EGFR expression in MM and representative solid tumor cell lines was further measured by RT-PCR. The results showed that EGFR1 and EGFR2, two major EGFR isoforms in cancers, were highly expressed in solid tumor cell lines, such as NSCLC cell line A549, breast cancer cell line H47D, cervical cancer cell line HeLa and prostate cancer cell line PC3, while neither was detected by RT-PCR in six MM cell lines including LP1 and RPMI-8226 (Figure 4D). This finding was consistent with previous studies that EGFRs are high in solid tumors (11), but it was not identified in RPMI-8226 cells (12). However, both RPMI-8226 and LP1 were sensitive to 4061 and 4065B, and these findings suggested that EGFR is dispensable for apoptosis induced by the conjugates of the endoperoxide and 4-anilinoquinazoline.

3.5. 4061 and 4065B inhibit the IGF1-R/AKT/mTOR signaling pathway

It is well known that growth factor IGF1 is a key stimulator to increase MM cell proliferation *via* its specific receptor IGF1-R. To find out the expression

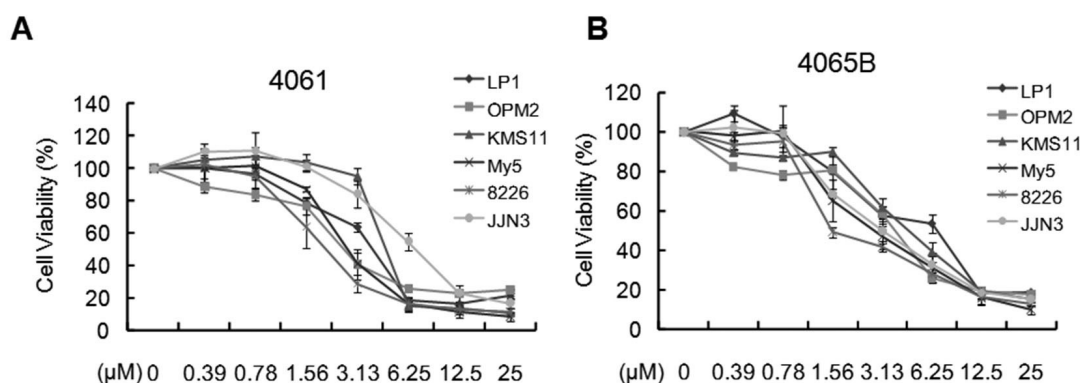


Figure 2. 4061 and 4065B inhibit MM cell proliferation. Various MM cell lines were treated with 4061 (A) or 4065B (B) at indicated concentrations for 72 hrs. Cells were then subjected to MTT assays as described in the Methods section.

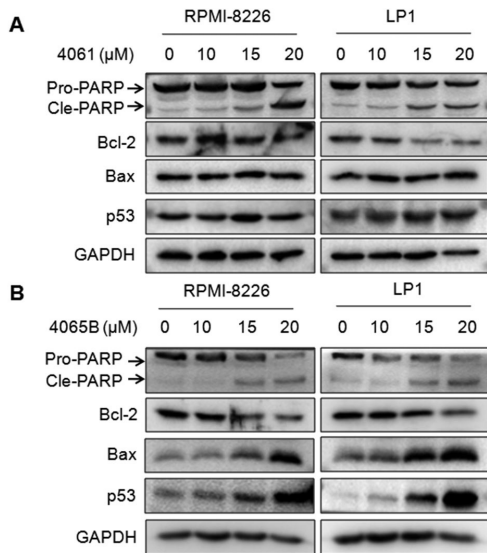


Figure 3. 4061 and 4065B activate apoptotic signaling in MM cells. RPMI-8226 and LP1 cells were treated with increasing concentrations of 4061 (A) and 4065B (B) for 24 hrs. Cell lysates were then prepared and subjected to immunoblotting assays against specific proteins as indicated. GAPDH was used as a loading control.

profile of IGF1-R, the mRNA levels of IGF1-R were analyzed by retrieving the Chapman Myeloma dataset based on 239 MM patients retrieved from the Oncomine Database (13). As shown in Figure 5A, compared with that from normal bone marrow cells, IGF1-R was significantly increased in MM cells and smoldering MM, an early stage of MM. Because IGF1/IGF1-R leads to the activation of the AKT/mTOR signaling pathway in MM survival (12,14), we wondered whether 4061 and 4065B could inhibit the AKT/mTOR pathway in MM cells stimulated by IGF1. To this end, starved LP1 and RPMI-8226 cells were treated with 4061 and 4065B or the known inhibitor BENC-511 (15), followed by stimulation with IGF1, a typical ligand of IGFR. The phosphorylation levels of IGFR1, AKT and mTOR kinases were strikingly increased by IGF1, but this activation was inhibited by both BENC-511 and the tested compounds 4061 and 4065B. Notably, 4061 and 4065B suppressed the activation of AKT and mTOR in a concentration-dependent manner but had no effects on their total protein expression (Figure 5B and 5C).

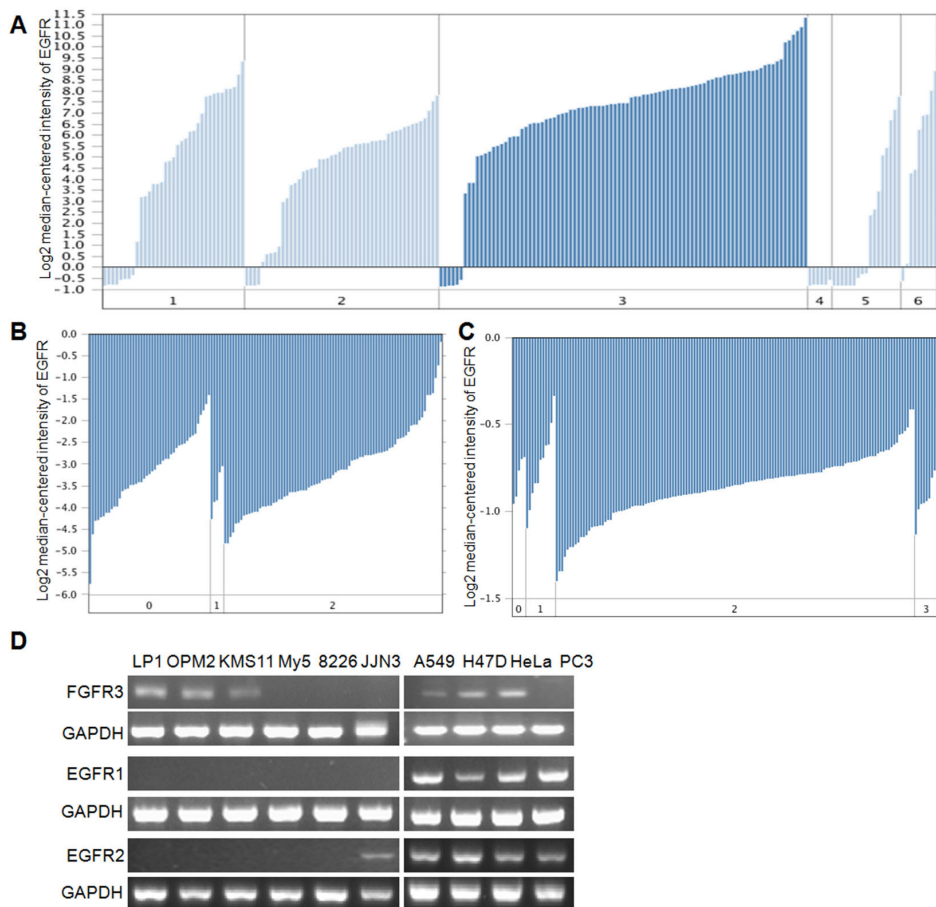


Figure 4. EGFR is not expressed in MM cells. (A) EGFR expression in various solid cancers and lymphoma retrieved from the Adai Cell Line dataset of Oncomine database. 1. Breast Cancer ($n = 35$); 2. Colorectal Cancer ($n = 48$); 3. Lung Cancer ($n = 91$); 4. Lymphoma ($n = 6$); 5. Melanoma ($n = 17$); 6. Ovarian Cancer ($n = 9$). (B) EGFR expression retrieved from the Zhan Myeloma dataset of Oncomine database. 0. normal ($n = 45$); 1. MGUS ($n = 5$); 2. MM ($n = 81$). (C) EGFR expression retrieved from the Agnelli Myeloma 3 dataset of Oncomine database. 0. Normal ($n = 5$); 1. MGUS ($n = 11$); 2. MM ($n = 133$); 3. plasma cell leukemia ($n = 9$). (D) Representative cancer cell lines were collected to isolate total RNA, followed by RT-PCR. The products were isolated on agarose gels.

Therefore, 4061 and 4065B displayed potent activity against MM by inhibiting IGF1-R/AKT/mTOR signaling transduction.

4. Discussion

The AKT/mTOR signaling pathway is activated by receptor tyrosine kinases including EGFR, FGFR, IGF1-R, PDGFR and others, upon individual ligand binding, thus this pathway plays a critical role in proliferation and survival of various cancers including solid tumors such as NSCLC and liquid tumors such as MM (16). Inhibitors of EGFR and IGF1-R have been developed for the treatment of various cancers (8,17). The most

well known one is gefitinib, the first-in-class EGFR inhibitor, which has been widely used for the treatment of NSCLC patients with mutated and overactivated EGFR. However, this powerful drug is not effective in most liquid tumors, such as MM (3). The present study found that the endoperoxide bridge conjugated to the 4-anilinoquinazoline core scaffold displayed potent anti-MM activity.

EGFR is the target of gefitinib in NSCLC but it is less expressed or not expressed in MM cells (18). The present study also demonstrated that EGFR1 and EGFR2, two major isoforms of EGFRs, were not detected from most MM cell lines examined, which is consistent with previous studies in which EGFR1 and other isoforms

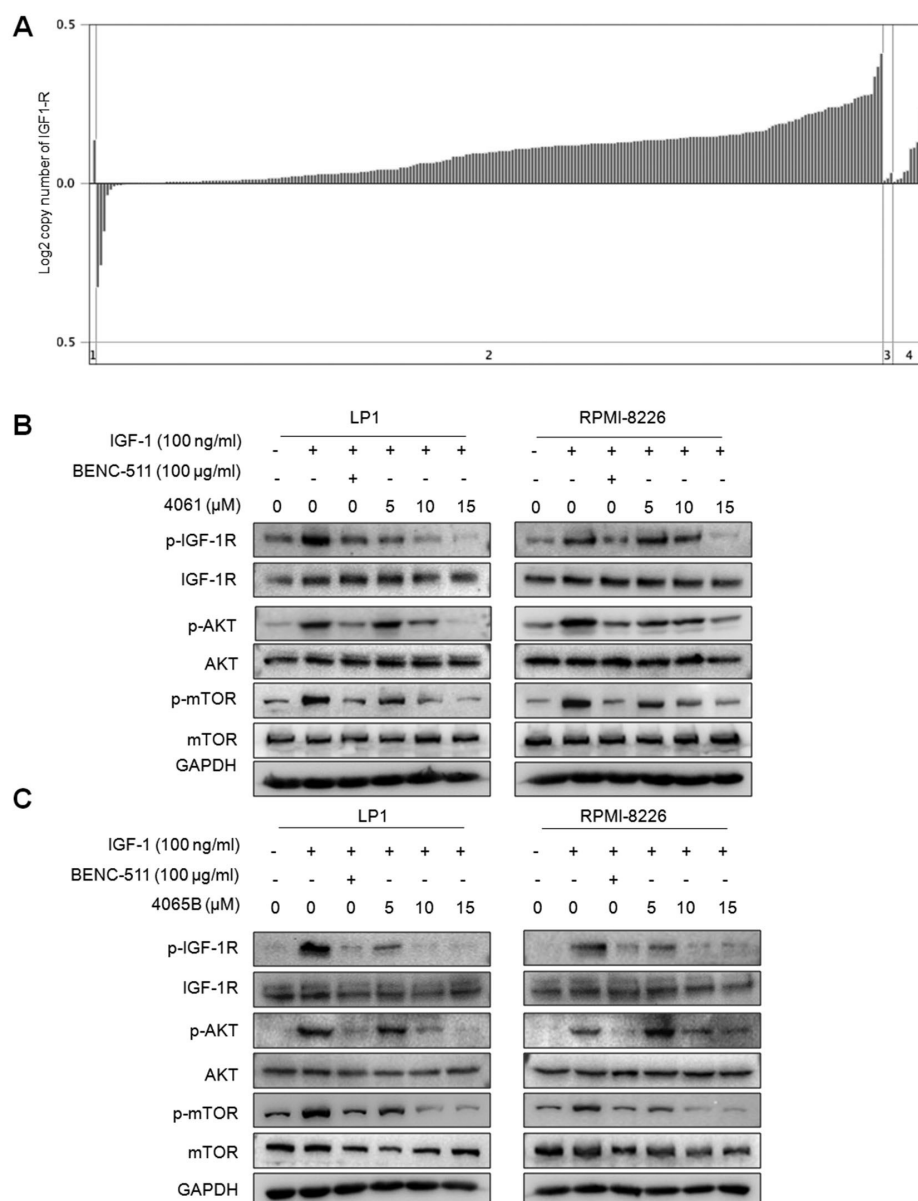


Figure 5. 4061 and 4065B inhibit the IGF1-R/AKT/mTOR signaling transduction. (A) The expression profile of IGF1-R in MM cells retrieved from the Chapman Myeloma 2 dataset of OncoPrint Dataset. 1. MGUS ($n = 2$); 2. MM ($n = 239$); 3. plasma cell leukemia ($n = 3$); 4. SMM ($n = 10$). (B, C) RPMI-8226 and LP1 were stored with 4061 (B) or 4065B (C) overnight or BENC-511 for 2 hrs. Cells were further treated with 100 µg/mL of IGF-1 for 15 min. Cells were then harvested for immunoblotting assays against indicated antibodies. GAPDH was measured as an internal control.

are not detected in RPMI-8226, a typical MM cell line (18) or at a relative low level compared with solid cancers (19). Therefore, there is no wonder that gefitinib is ineffective in MM. However, 4061 and 4065B that conjugate 4-anilinoquinazoline, the pharmacophore of gefitinib, and peroxide bridge, the pharmacophore of artemisinin, displayed great activity in MM cell apoptosis. The underlying mechanisms of these two compounds in MM cell apoptosis are probably associated with the production of reactive oxygen species (ROS). ROS is believed to be a major mechanism in the anti-cancer activity of some compounds such as artemisinin and derivatives that contain an endoperoxide group (20). The *in vivo* studies demonstrated that the ferrous iron mediated cleavage of the endoperoxide group to release ROS and/or carbon centered radicals that further induce DNA damage, mitochondrial depolarization and apoptosis. In the RTK-associated signaling transduction, IGF1/IGF1-R activated AKT/mTOR signaling pathway is probably another target because activation of IGF1-R by phosphorylation is inhibited by both 4061 and 4065B. The IGF1/IGF1-R pathway including its constitutive ligands and receptors not only contributes to the survival, proliferation, and homing of MM cells, but also leads to MM-associated angiogenesis and osteolysis (21,22). IGF1/IGF1-R overexpression is closely correlated to poor prognosis in MM patients (22). Targeting the IGF system has been developed for MM treatment, *e.g.* IGF1R monoclonal antibodies such as AVE1642 (23) and small chemical compound inhibitors OSI-906 (24) have been developed in preclinical models with great potential for MM treatment. In the present study, the growth factor IGF1 drastically induces the phosphorylation of both AKT and mTOR, but these signals were suppressed by both 4061 and 4065B in a concentration-dependent manner, suggesting that 4061 and 4065B at least inhibit IGF1/IGF1-R-mediated AKT/mTOR signaling. This is probably one major mechanism in that 4061 and 4065B go beyond gefitinib that is not effective in MM cell apoptosis.

In conclusion, this present study reported novel agents by conjugating endoperoxide bridge to 4-anilinoquinazoline that display potential for the treatment of liquid cancers such as MM by suppressing the IGF1-R/AKT/mTOR signaling pathway. These compounds might represent a novel class of anti-MM drugs.

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