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Molecular Target Therapy against Neuroblastoma

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Abstract

Neuroblastoma, originated from neural crest cells, is the most common extracranial solid tumor in childhood. Treatment is of limited utility for high-risk neuroblastoma and prognosis is poor. The high incidence of resistance of advanced-stage neuroblastoma to conventional therapies has prompted investigators to search for novel therapeutic approaches. Activation of IGF-R/PI3K/Akt/mTOR signaling pathway correlates with oncogenesis, poor prognosis, and chemotherapy resistance in neuroblastoma. Therefore, we investigated the effect of IGF-R/PI3K/Akt/mTOR signaling inhibitors in neuroblastoma. Significantly, IGF-R/PI3K/Akt/mTOR signaling inhibitors effectively inhibited cell growth and induced cell cycle arrest, autophagy, and apoptosis in neuroblastoma cells. Moreover, IGF-R/PI3K/Akt/mTOR signaling inhibitors significantly reduced tumor growth in mice xenograft model without apparent toxicity. Therefore, these results highlight the potential of IGF-R/PI3K/Akt/mTOR signaling pathway as a promising target for neuroblastoma treatment. Therefore, IGF-1R/PI3K/Akt/mTOR signaling inhibitors should be further investigated for treatment in clinical trials for high-risk neuroblastoma.

Keywords: neuroblastoma, insulin-like growth factor (IGF), phosphatidylinositol 3-kinase (PI3K), protein kinase-B (Akt), mammalian target of rapamycin (mTOR)

1. Introduction

Neuroblastoma (NB) is one of the most common extracranial solid tumors of early childhood [1, 2]. Prognosis of patients with NB depends on tumor stage, patient's age, and biologic feature of the tumor cells [3]. In patients under 1 year of age, NB is curable and sometimes spontaneously regress [4]. However, in older children with advanced stage, often the tumor is very aggressive, and patients have poor prognosis despite treatment with high-dose chemotherapy combined with autologous hematopoietic stem cell transplantation. Although immunotherapeutic therapy such as anti-GD2 monoclonal antibody has improved outcomes of advanced stage of NB, a number of patients still relapse and eventually die of disease [5]. The high incidence of resistance of high-risk NB to conventional therapies has prompted us to search for novel therapeutic approaches.

It was reported that phosphorylated protein kinase-B (Akt) correlates with poor patients' prognosis in NB [6], and the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway has subsequently been linked to augmented cell survival [7] and increased resistance to chemotherapy in NB [8].

Therefore, targeting the PI3K/Akt/mTOR signaling pathway by appropriate inhibitors appears to be a promising strategy for overcoming therapy resistance [9].

Here, we demonstrate that NB cell lines are heterogeneous in their insulin growth factor-1 (IGF-1) receptor-mediated signaling [10]. The pattern of IGF-1 receptor/PI3K/Akt/mTOR pathway-mediated proliferation is an important determinant of the response to IGF-1 receptor antagonistic therapy in human NB [10]. Furthermore, our results highlight the potential of IGF-1 receptor/PI3K/Akt/mTOR signaling pathway as a promising target for NB treatment [10–13].

2. Heterogeneity of IGF-1 receptor/Akt pathway-mediated proliferation in NB

IGF-1, IGF-2, and insulin belong to a family of mitogenic growth factors and are involved in normal growth and differentiation of most tissues. The biological actions of both IGFs and insulin can be mediated by the IGF-1 receptor which is involved in mitogenic, anti-apoptotic, and oncogenic transforming responses [14, 15]. The IGF-1 receptor has two extracellular α -subunits and two intracellular β -subunits that form a heterotetrameric complex. Ligand interaction with α -subunits triggers the autophosphorylation of tyrosine kinase domains within the β -subunit [16–18]. The tyrosine kinase domains are connected to several intracellular pathways such as PI3K/Akt [19, 20]. Dysregulation of the IGF-1 receptor pathway is involved in promoting oncogenic transformation, cell proliferation, metastasis, angiogenesis, and resistance in numerous malignant diseases, such as multiple myeloma [21], carcinomas [22], and NB [23]. Since high cellular heterogeneity is a hallmark of NB, which may account for the wide range of clinical presentations and nonuniform response to treatment, we hypothesized that NB cells are heterogeneous in their IGF-1 receptor signaling-mediated cell proliferation. Thirty-one NB cell lines were cultured in three different conditions, insulin-containing serum-free medium (SFM), RPMI1640 without FBS (serum starvation medium), and RPMI1640 with 10% FBS (serum-containing medium). Based on the response patterns, 31 cell lines were subdivided into three groups [10]. Group 1, which consisted of three NB cell lines, could proliferate for more than 3 days in SFM, RPMI1640 without FBS, and RPMI1640 with 10% FBS [10]. Group 2, which consisted of 10 cell lines, could proliferate in SFM and RPMI1640 with 10% FBS but not in RPMI1640 [10]. Group 3, which consisted of 18 NB cell lines, proliferated only in RPMI1640 with 10% FBS [10]. NB cell proliferation in RPMI1640 in the presence of exogenous IGF (IGF-1, IGF-2) and insulin was examined. These IGF and insulin accelerated cell proliferation in group 1 and group 2 NB cell lines but not in group 3 NB cell lines [10]. Group 1 NB cell lines were able to proliferate in RPMI1640 in the absence of exogenous IGF and insulin [10].

3. Impairment of Akt activation and cell proliferation in NB by IGF-1 receptor inhibitor, picropodophyllin (PPP)

The IGF-1 receptor inhibitors, such as IGF-1 receptor-neutralizing antibodies and IGF-1 receptor antisense/siRNA, have been shown to block cancer cell proliferation [24]. Selective IGF-1 receptor inhibitor, picropodophyllin (PPP), lacks inhibitory activity on tyrosine phosphorylation of insulin receptor tyrosine kinase (RTK) [25]. Inhibition of the IGF-1 RTK with PPP is noncompetitive with respect to ATP, suggesting interference with the IGF-1 receptor at substrate level [26]. It is reported that PPP specifically blocks phosphorylation of the Tyr1136 residue in the activation loop

of IGF-1 receptor kinase [27]. Inhibition of IGF-1 receptor with PPP has been demonstrated in a lot of cancers such as multiple myeloma [26], melanoma [28], breast cancer [29], and glioblastoma cells [30]. PPP inhibited Akt activation and suppressed cell proliferation in group 1 and group 2 NB cell lines but less in group 3 NB cell lines [10]. Elevation of ERK phosphorylation was only observed in group 1 NB cell lines [10]. U0126 is a MEK inhibitor effectively suppressed ERK phosphorylation, U0126 (2.5 μ M) did not suppress cell proliferation induced by IGF-1, IGF-2, or insulin in group 1 and group 2 NB cell lines [10]. In groups 1 and 2 NB cell lines, IGF-1 receptor/PI3K/Akt pathway is critical for cell proliferation. Although IGF-1, IGF-2, and insulin activated Akt in group 3 NB cell line, cell proliferation was not increased in RPMI1640. This suggests that the activation of IGF-1R/Akt pathway is insufficient for cell proliferation [10]. Since IGF-1 and IGF-2 regulate apoptosis [31] and cell cycle progression [32], activation of caspase 3 and PARP was examined in NB cell lines. In group 1 NB cell lines, caspase 3 and PARP were not cleaved in RPMI1640 [10]. In group 2 NB cell lines, caspase 3 and PARP were cleaved in RPMI1640, and the cleavage of caspase 3 was suppressed by addition of IGF-1, IGF-2, and insulin [10]. In group 3 NB cell lines, cleavages of caspase 3 and PARP were observed in RPMI1640 [10]. However, they were not suppressed by IGF-1, IGF-2, and insulin, even though Akt activation was upregulated by IGF-1, IGF-2, and insulin [10]. Since PPP induced G2/M arrest and apoptosis by inhibiting IGF-1 receptor [26, 33], cell cycle phase distribution in NB cells was examined in the presence of PPP. PPP treatment (2.5 μ M) for 12 hours increased the G2/M fraction and shifted cell cycle profile from G0-G1 dominant to G2/M dominant in group 1 and group 2 NB cell lines [10]. However, group 3 NB cell lines did not show G2/M arrest and the G0/G1 fraction was not affected [10]. Furthermore, cyclin B1, a marker protein of G2/M phase of cell cycle, was upregulated, and cyclin D1, a marker protein of G0/G1, synchronously declined in group 1 and group 2 NB cell lines [10]. However, accumulation of cyclin B1 and decline of cyclin D1 were not observed in group 3 NB cell lines [10]. This may be explained by the insensitiveness of group 3 cell lines to PPP-induced G2/M arrest.

4. Impairment of Akt activation and cell proliferation in NB by Akt inhibitor, MK2206

MK2206 selectively inhibits AKT and has potency against Akt1 and Akt2 isoforms than Akt3. In pediatric solid tumors, MK2206 is effective in vitro and in vivo [34, 35]. In clinical trials, stable disease was observed in different kinds of cancers [36, 37]. Our results also suggested that MK2206 (2.5 μ M) completely inhibited Akt phosphorylation and cell proliferation in NB cell lines [10]. Furthermore, MK2206 also impaired the cell proliferation induced by exogenous IGF and insulin in NB cell lines [10].

5. Inhibition of NB cell proliferation by mTOR inhibitor

The PI3K/Akt/mTOR signaling cascade is one of the most important intracellular pathways, which is frequently activated in diverse cancers [38, 39]. The PI3K/Akt/mTOR pathway can be activated by transmembrane tyrosine kinase growth factor receptors, such as IGF-1 receptor, fibroblast growth factor receptors, ErbB family receptors, and others [40, 41]. Both mTOR S2448 and mTOR S2481 were extensively phosphorylated in NB cell lines [13]. Cell proliferation of NB cell lines was inhibited by AZD8055, a potent dual mTORC1-mTORC2 inhibitor [13]. According to the IC₅₀, the NB cell lines were divided into two groups, sensitive to AZD8055 group (IC₅₀ < 0.5 μ M) and insensitive to AZD8055 group (IC₅₀ > 0.5 μ M) [13]. We also found

that insensitive group showed lower mTOR ($p < 0.001$) expression and lower activity of mTOR complex 1 ($p = 0.013$) and mTOR complex 2 ($p = 0.023$) [13]. Cell cycle distribution analysis of NB cell lines was performed by flow cytometry. Cell cycle was affected with an increase in G0/G1 phase in dose-dependent manner of AZD8055 [13]. Western blotting analysis revealed that Cyclin D1 and Cyclin D3 were downregulated in AZD8055-treated NB cells [13]. AZD8055 inhibited both mTOR S2448 and mTOR S2481 phosphorylation significantly in a concentration-dependent manner [13]. Phosphorylation of downstream targets of mTOR complex 1, P70S6K T389 and 4E-BP1 S65, was also inhibited by AZD8055 treatment [13]. AZD8055 inhibited mTOR complex 2 substrates, Akt S473 and Akt T308 [13]. Although phosphorylation of Akt S473 was persistently inhibited in response to AZD8055 treatment, phosphorylation of Akt at the T308 site was inhibited for only 3–6 hours. These results indicate that AZD8055 inhibits mTOR activity and its downstream proteins *in vitro* in NB cells. Interestingly, NB cell lines were induced autophagy by AZD8055 treatment via downregulation of Akt/mTOR signaling pathway [13]. Autophagy inhibitor, 3-methyladenine, treatment resulted in a significant decrease of the AZD8055-induced apoptosis [13]. These results suggest that AZD8055 inhibited cell growth and induced cell cycle arrest, autophagy, and apoptosis in NB cells. Moreover, NB tumor growth in athymic nude mice was significantly inhibited by AZD8055 without toxicity [13]. Taken together, our results highlight that mTOR is a promising target for NB treatment. AZD8055 might be investigated for treatment of patients with advanced and refractory NB.

6. AZD8055 treatment in combination with MEK/ERK inhibitor

The problem that will plague single-target drugs is the cancer's ability to activate alternative survival pathways leading to drug resistance and toxicity even in the multimodality setting. In addition, activation of multiple signaling pathways effectively causes cancer cell proliferation and survival. For example, RAS and PI3KCA are concurrently activated in melanoma, lung, and colorectal cancers [42–44]. These results suggested that combination therapies targeted on multiple signaling pathways could be more effective than targeting either pathway alone. Although AZD8055 is a promising drug in NB treatment [13], AZD8055-resistant NB sublines were acquired by prolonged stepwise exposure. After incubation with AZD8055 for 4–12 weeks, two acquired AZD8055-resistant sublines proliferated stably in RPMI1640 plus 10% FBS medium in the presence of AZD8055 (3 μ M) [12]. The AZD8055-resistant sublines exhibited marked resistance to AZD8055 compared to the parent cells, and IC_{50} of the resistant sublines was 60–100 times higher than the parent NB cell lines [12]. By cell cycle analysis, accumulation of S phase was observed, and cyclin D3 and CDK4 were upregulated in AZD8055-resistant sublines [12]. Although AZD8055 treatment inhibited MEK/ERK activation in parent cells, MEK/ERK phosphorylation was continued despite AZD8055 treatment in resistant cells [12]. The combination therapy of AZD8055 and MEK/ERK inhibitor U0126 significantly inhibited cell proliferation compared to U0126 monotherapy [12], suggesting that combination therapy can overcome AZD8055 resistance. Furthermore, in athymic mice model, AZD8055 and U0126 co-treatment was more efficient to suppress resistant NB tumor growth compared to U0126 monotherapy [12].

7. MK2206 treatment in combination with PDK1 inhibitor

MK2206 treatment induced a dose-dependent inhibition of cell proliferation, with IC_{50} ranging from 1.22 to 4.35 μ M in NB cell lines [11]. MK2206-resistant cells

were induced by stepwise escalation of MK-2206 exposure (4–12 weeks) [11]. These cells proliferated in RPMI1640 plus 10% FBS medium in the presence of MK2206 (5 μ M), while cell death was induced in parent cells [11]. IC₅₀ of the resistant cell lines was 6–7 times higher than the parent NB cell lines [11]. Small-molecule GSK2334470 selectively inhibits 3-phosphoinositide-dependent protein kinase 1 (PDK1) with low concentration but does not suppress the activity of other protein kinases at higher concentrations [45]. Although GSK2334470 attenuated cell proliferation in both parent cells and MK2206-resistant sublines, IC₅₀ of GSK2334470 in resistant sublines was lower than that of parent cell lines [11]. GSK2334470 induced G0-G1 accumulation of cell cycle phase distribution in parent cell lines [11]. In MK2206-resistant sublines, G0-G1 accumulation induced by GSK2334470 was higher than parent cell lines [11].

8. Effect of NB MYCN status on susceptibility to AZD8055

Amplification of the MYCN oncogene is the most powerful single predictor of adverse outcome of NB [46]. MYCN amplification is observed in about 20% of all NB patients and is usually associated with fatal outcome of the disease [47]. Schramm et al. demonstrated transcriptomal upregulation of mTOR-related genes by MYCN [48]. MYCN-driven NB in mice displayed activation of the mTOR pathway on the protein level, and activation of MYCN in NB cells resulted in high sensitivity toward mTOR inhibition [48]. Therefore, it is examined whether MYCN

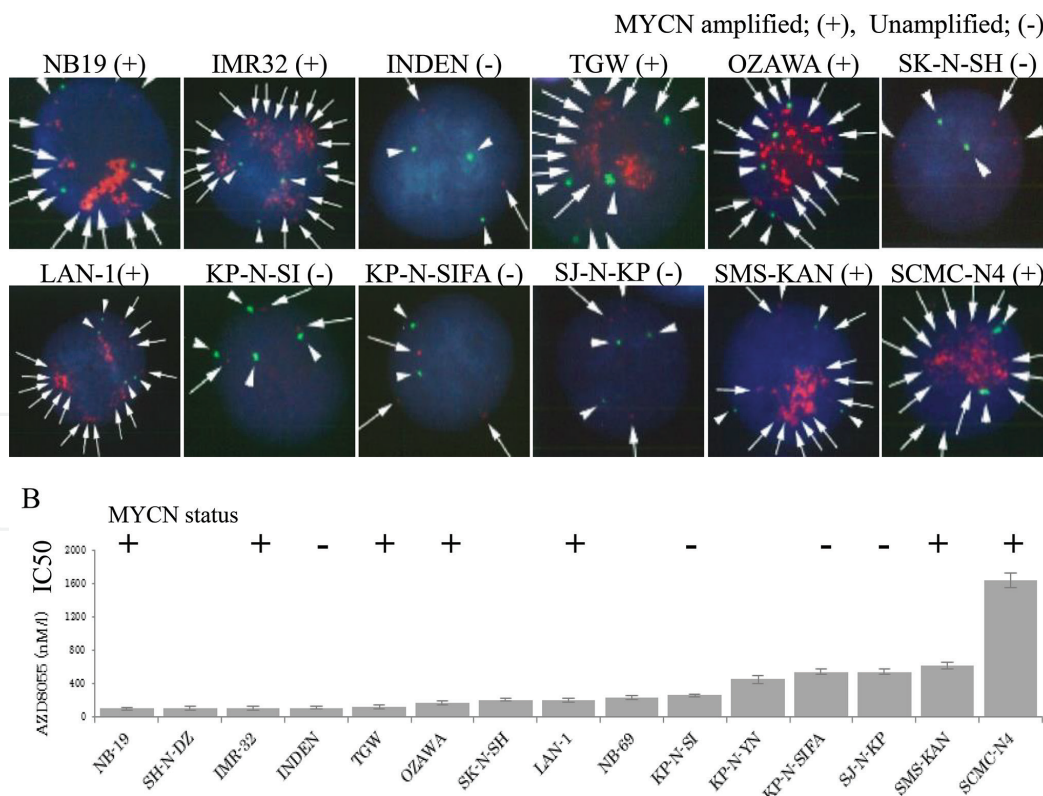


Figure 1. Effect of MYCN status on susceptibility to AZD8055. (A) MYCN gene in tested 12 NB cell lines. MYCN status was defined as previously [53]. No alteration: cells with two MYCN signals and two CEP2 signals; amplification: the number of MYCN signals is at least 10 copies greater than the control probe signals; loss/imbalance: presence of at least two MYCN signals and increased CEP2 signals; gain: the number of MYCN signals is 1–9 copies more than CEP2 signals. (B) Fifteen NB cell lines were treated with AZD8055 at different concentrations in RPMI1640 + 10% FBS. Cell growth was evaluated as cell numbers at 72 hours. Data are expressed as the mean \pm SD. IC₅₀ (half maximal inhibitory concentration) of the 15 NB cell lines was calculated depending on the MTT results.

status of NB cell lines affects susceptibility to AZD8055. Using fluorescence in situ hybridization (FISH), we observed MYCN amplification in all investigated nuclei of NB19, IMR32, TGW, OZAWA, LAN-1, SMS-KAN, and SCMC-N4 cell lines (**Figure 1A**). As shown in **Figure 1A**, MYCN amplification was not observed in INDEN (loss/imbalance), SK-N-SH (gain), KP-N-SI (loss/imbalance), KP-N-SIFA (gain), and SJ-N-KP (loss/imbalance). As shown in **Figure 1B**, the relationship between MYCN status and susceptibility to AZD8055 was not found.

9. ALK as a therapeutic target in NB

Constitutive activation of the ALK receptor tyrosine kinase by mutation or translocation appears to contribute to the malignant phenotype of several cancers, including NB, making it a potentially therapeutic target [49]. Both wild type ALK and the F1174L-mutated ALK upregulate MYCN expression [50], and these two genes lead to constitutive phosphorylation of ALK and of downstream signaling molecules, PI3K/AKT/mTOR, that are critical for cell proliferation and survival [51]. ALK^{F1174L} cosegregates with MYCN amplification in patients, and this combination is associated with a particularly poor prognosis, as shown by the fatal outcome of 9 of 10 children with ALK^{F1174L}/MYCN-amplified tumors [52]. Targeting ALK with PI3K/Akt/mTOR inhibitors may inhibit cell growth in tumor lines with concomitant MYCN amplification.

10. Conclusions

NB cell lines can be categorized into three groups by the patterns of IGF-1R/Akt pathway response. PPP, MK2206, and AZD8055 showed significant antitumor effect in NB cells. Our current results highlight the potential of IGF-1R/PI3K/Akt/mTOR pathway as a promising target for NB treatment. PPP, MK2206, and AZD8055 should be further investigated for NB treatment in clinical trials.

Conflict of interest

The authors declare no potential conflicts of interest.

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