Cell Survival Signaling in Neuroblastoma

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Abstract

Neuroblastoma is the most common extracranial solid tumor of childhood and is responsible for over 15% of pediatric cancer deaths. Neuroblastoma tumorigenesis and malignant transformation is driven by overexpression and dominance of cell survival pathways and a lack of normal cellular senescence or apoptosis. Therefore, manipulation of cell survival pathways may decrease the malignant potential of these tumors and provide avenues for the development of novel therapeutics. This review focuses on several facets of cell survival pathways including protein kinases (PI3K, AKT, ALK, and FAK), transcription factors (NF-κB, MYCN and p53), and growth factors (IGF, EGF, PDGF, and VEGF). Modulation of each of these factors decreases the growth or otherwise hinders the malignant potential of neuroblastoma, and many therapeutics targeting these pathways are already in the clinical trial phase of development. Continued research and discovery of effective modulators of these pathways will revolutionize the treatment of neuroblastoma.

Neuroblastoma

Neuroblastoma is the most common extracranial solid tumor of childhood. This neuroendocrine tumor is most commonly located in the adrenal medulla, but may arise anywhere that sympathetic neural tissue is found. Over 50% of these tumors occur in children under 2 years of age[1], and neuroblastoma is responsible for over 15% of pediatric cancer deaths.[2] Despite recent advances in chemotherapy and surgical care, this tumor continues to carry a dismal prognosis for children presenting with advanced or metastatic disease, with a long term survival of only 18–30%.[3] Neuroblastoma tumorigenesis and malignant transformation is driven by overexpression and dominance of cell survival pathways and a lack of normal cellular senescence or apoptosis. Therefore, manipulation of cell survival pathways may decrease the malignant potential of these tumors and provide avenues for the development of novel therapeutics. The current review will discuss some of the common cell survival signaling pathways in neuroblastoma.

Neuroblastoma and Kinases

One group of proteins involved in neuroblastoma cell survival pathways are kinases. Kinases serve to phosphorylate, and thereby activate, other factors in the cell signaling pathway. A well-known kinase pathway involved in cellular survival is the c-AKT kinase cascade. The AKT cascade is important in tumorigenesis as it controls inhibition of normal programmed cell death.[4] Central to this cascade is AKT, a serine/threonine kinase that regulates many cellular functions including cell growth[5], proliferation[6], survival[7], and angiogenesis.[8] AKT is activated and recruited to the plasma membrane by phosphatidylcholine 38-OH (PI3K), a kinase stimulated by growth factors. Once at the plasma membrane, other kinases such as 3-phosphoinositide-dependent protein kinases

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(PDKs) phosphorylate AKT[4], resulting in an active moiety.[9,10] Many studies have shown the effects of AKT in reducing apoptosis in a variety of cell lines. Dudek et al showed that cerebellar neurons transfected with HA-AKT vector overexpressing AKT, had reduced apoptosis following withdrawal of growth factors.[11] Khwaja et al showed decreased apoptosis following cell matrix detachment in kidney epithelial cells transfected with a constitutively active form of AKT.[12]

The PI3K-AKT cell survival pathway has been demonstrated to be important in many human cancers[13] including neuroblastoma.[14] AKT phosphorylation has been noted in a number of human neuroblastoma cell lines including SK-N-SH, SH-SY5Y, SK-N-BE, SH-EP, and IMR-32.[15] In human tissue specimens, phosphorylation of AKT was more abundant in primary neuroblastoma samples than in benign ganglioneuromas or normal adrenal tissue.[14] In addition, AKT phosphorylation correlated with advanced stage of disease, unfavorable histology and amplification of the *MYCN* oncogene in human neuroblastoma specimens.[15] Upregulation of AKT has resulted in more aggressive neuroblastoma cell lines. One study treated human neuroblastoma cells with bilobalide, which increased phosphorylation of PI3K as well as AKT, prior to exposing them toxic stimuli. The cells with upregulated PI3K/AKT showed significantly less apoptosis when compared to cells with normal PI3K/AKT activity.[16] In another example, SH-SY5Y neuroblastoma cells exposed to oxidative stress were protected from cell death when treated with *Lonicera japonica*, which enhanced AKT phosphorylation.[17]

A number of methods have been described to downregulate AKT in neuroblastoma cell lines, in attempts to increase apoptosis and cell death. In one study, long-term exposure of SH-SY5Y cells to interferon-β decreased activation of the PI3K-AKT pathway and increased apoptosis.[18] Another method of AKT downregulation involved mTOR inhibition with rapamycin. mTOR is a serine/threonine kinase that is a downstream effector of AKT, regulating cell growth, proliferation, cytoskeleton organization, and energy metabolism.[19,20,13] Johnsen and colleagues showed that rapamycin inhibition of mTOR in neuroblastoma cell lines had anti-proliferative effects.[14] When rapamycin was administered to mice bearing SH-SY5Y xenografts, there was decreased tumor growth, increased apoptosis, decreased cell proliferation, and decreased small vessel density in the tumors. Finally, they showed that cell lines expressing high levels of MYCN were more sensitive to rapamycin treatment.[14] In another study, small molecule inhibitors of the PI3K/AKT pathway, OSU03012 and PI103, were utilized to treat neuroblastoma cell lines and established subcutaneous xenografts in mice. These small molecules both downregulated the phosphorylation of AKT and resulted in decreased cell survival *in vitro* and decreased tumor growth *in vivo*. Again, the findings were more pronounced in cell lines with *MYCN* amplification.[21] These observations were important as amplification of the *MYCN* oncogene is the most significant adverse prognostic indicator in neuroblastoma.[22,23]

All these findings lend evidence to the important role the PI3K/AKT pathway plays in neuroblastoma cell survival and tumorigenesis. Modulation of this pathway may be an important target for future chemotherapeutic agents in the treatment of neuroblastoma. In fact, current ongoing studies include several phase I clinical trials involving AKT inhibitors. The Children's Oncology Group has an open protocol for a phase I study of temsirolimus (Table 1), a rapamycin analog, in combination with irinotecan and temozolomide for relapsed or refractory pediatric solid tumors including neuroblastoma. Perifosine (Table 1) is a synthetic oral alkylphospholipid that targets the lipid-binding PH domain of AKT, inhibiting the translocation of AKT to the plasma membrane.[24] Recent studies utilizing perifosine in combination with temsirolimus have shown good results in some solid malignancies in adults[25], and perifosine has been shown to increase the sensitivity of neuroblastoma cells to chemotherapeutic agents.[26] Although the safety of perifosine has not

yet been established in children, this drug may prove to be an adjunctive agent for neuroblastoma therapies.

Anaplastic lymphoma kinase, ALK, is another kinase involved in neuroblastoma cell survival signaling. Originally discovered in anaplastic large cell lymphoma cells, ALK is part of the insulin receptor family of trans-membrane receptor tyrosine kinases[27] involved in cell growth and development, particularly in the neuronal and central nervous system.[28] It has been identified in several tumor types including inflammatory myofibroblastic tumors[29], non-small cell lung cancer[30,31], diffuse large B-cell lymphoma[32], squamous cell cancer of the esophagus[33,34], and neuroblastoma. In neuroblastoma, the ALK protein has been found to be expressed in over 90% of tumor samples examined[35], and expression of the ALK protein was associated with mutations in the *ALK* gene.[36] In fact, *ALK* gene mutations have been linked to familial as well as sporadic neuroblastoma. Mosse et al, using whole-genome scans of neuroblastoma pedigrees, showed that heritable mutations of *ALK* were the main cause of familial neuroblastoma. They also showed that somatically acquired mutations occurred in 12.4% of the tumors and cell lines tested, and that the presence of an aberrant *ALK* copy number was associated clinically with metastasis at diagnosis and death from disease.[37] Furthermore, knockdown of *ALK* with siRNA resulted in significant inhibition of growth in neuroblastoma cell lines with mutant or amplified *ALK*.[37] In a 2010 meta-analysis of neuroblastoma tumors, *ALK* gain-of-function mutations were found to be present in 6.9% of tumors, and the most common *ALK* mutation (F1174) was closely associated with *MYCN* amplification (58.8%) versus tumors with wild-type *ALK*.[38] Recently, *ALK* has also been found to be an initiator of *MYCN* transcription, regulating the *MYCN* promoter in CLB-GA, CLB-GE, CLB-BAR, and IMR-32 neuroblastoma cell lines.[39]

Recent studies have shown that manipulation of ALK can be used as a treatment target for neuroblastoma. One group found that neuroblastoma cell lines with mutated *ALK* and overactive ALK expression had greater cell death than those with non-mutated *ALK* when treated with the glycosylation inhibitor, tunicamycin. Downstream survival signaling products of ALK including AKT, ERK1/2, and STAT3 were decreased and apoptosis was activated after treatment with tunicamycin.[40] Schonherr et al also found that the neuroblastoma cell lines overexpressing ALK were susceptible to treatment with ALK inhibitors. Treatment of cells with the small molecule ALK inhibitors crizotinib (PF-2341066, Table 1) or NVP-TAE684 (Table 1), and small interfering RNA against *ALK*, led to decreased cell proliferation and downregulation of *MYCN* transcription.[39] These results demonstrate that ALK may be another kinase to target for innovative neuroblastoma therapies. To that end, patients are currently being accrued in a Phase 1/2 trial being conducted by the Children's Oncology Group studying the oral small molecule ALK inhibitor, crizotinib, in children with relapsed or refractory neuroblastoma and other solid tumors.

A third kinase involved in neuroblastoma cell survival signaling is focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase found on the cellular periphery in focal adhesions, and is a key regulator of pathways involved in proliferation, viability, and survival.[41] Recently, it was shown that FAK was present in a number of human tumors including breast cancer and melanoma cells.[42,43,44,45] FAK inhibition results in decreased cellular survival. For example, treatment of breast cancer cells (BT474 and MCF-7) with adenoviral gene transduction of a dominant-negative FAK protein (AdFAK-CD) resulted in loss of cell adhesion, degradation of native FAK, and induction of apoptosis while leaving normal mammary cells unaffected.[42]

FAK has been found to be overexpressed in neuroblastoma cell lines.[46] It was also shown that FAK was present in human neuroblastoma tissues and the expression of FAK was

related to tumor stage.[47] In neuroblastoma, it was demonstrated with chromatin immunoprecipitation studies, electrophoretic mobility shift and dual luciferase assays, that MYCN bound to the FAK promoter and functioned as a transcription factor for FAK.[46] Golubovskaya et al demonstrated that NF-κB is involved in the regulation of FAK transcription as well. Utilizing a luciferase assay, they showed that inhibition of NF-κB with a super-repressor of NF-κB resulted in decreased FAK transcriptional activation.[48]

The inhibition of FAK in neuroblastoma using small interfering RNAs resulted in FAK dephosphorylation and cellular phenotypic changes including decreased cell attachment and rounding, and an increase in apoptosis. The effects of FAK inhibition with siRNA were more pronounced in the MYCN positive cells with greater FAK expression.[46] When FAK expression was abrogated with a dominant-negative construct, AdFAK-CD, there was a significant decrease in cell survival, again more marked in MYCN+ cell lines.[49] Other methods of FAK inhibition have been studied in neuroblastoma and they include small molecules targeting FAK or its phosphorylation, such as NVPTAE-226 (Table 1), or 1,2,4,5, benzenetetraamine tetrahydrochloride (Table 1). Treatment of neuroblastoma cell lines with NVPTAE-226 led to decreased neuroblastoma cell survival, increased apoptosis, and G_2 cell cycle arrest.[50] Another small molecule, 1,2,4,5, benzenetetraamine tetrahydrochloride (Y15), has also been shown to decrease neuroblastoma growth *in vitro*.[51] In addition, this study also showed a significant decrease in tumor growth in a mouse xenograft model.[51] There are currently two Phase 1 clinical trials investigating FAK inhibitors for the treatment of cancer.[52] None involve children, but clearly targeting FAK may provide a novel treatment for this disease.

Neuroblastoma and Transcription Factors

Cell survival in neuroblastoma may also be regulated by activation of genes that promote survival or inhibit apoptosis. NF-κB is a transcription factor that is ubiquitously expressed, but is sequestered in the cytoplasm bound to inhibitor of κB (IκB) proteins.[53] A variety of stimuli induce kinases that phosphorylate IκB, resulting in their degradation and freeing the NF-κB.[54] Upon activation, NF-κB translocates into the nucleus where it binds to promoters of several survival genes including Bcl-2 and caspase inhibitors.[4] Uninhibited activity of NF-κB was shown to lead to malignant transformation of NIH3T3 fibroblasts as measured by increased cell saturation density, increased growth in soft agar, and increased tumor growth in nude mice.[55] NF-κB signaling has also been implicated in the inhibition of TNF-induced apoptosis in mouse fibroblasts. Fibroblasts with knockout of the p65 subunit of NF-κB were significantly more sensitive to apoptosis following TNF treatment, showing that active NF-κB had a protective effect on the cells.[56] Finally, stimulation of multiple myeloma cells with insulin-like growth factor led to increased nuclear translocation of NF-κB and DNA binding activity, and increased resistance to TRAIL-induced apoptosis.[57]

NF-κB has been shown to be present and inducible in neuroblastoma cell lines.[58] Russo et al showed that in glucose deprived neuroblastoma cells, insulin-like growth factor-1 led to increased transcription of NF-κB, which in turn promoted cell survival in the low glucose environment.[59] NF-κB has also been implicated in neuroblastoma chemoresistance, as doxorubicin and VP16 have both been shown to trigger NF-κB activation in neuroblastoma cells, inhibiting apoptosis.[60] In contrast, inhibition of NF-κB in neuroblastoma cell lines resulted in increased cell death. Karacay et al showed that inhibition of NF-κB in SK-N-SH neuroblastoma cells via adenoviral transfection of a dominant-negative mutant, Ad5-IKKβKA, resulted in significant cell death when the cells were exposed to an active TRAIL vector.[61] Similarly, Ammann et al showed that inhibition of NF-κB activation by overexpression of a dominant-negative mutant IκBα-super-repressor resulted in sensitization of SH-EP neuroblastoma cells to TRAIL-induced apoptosis.[60] Gao et al

utilized synthetic oleanolic triterpenoid to inhibit cell growth and induce apoptosis in SK-N-MC neuroblastoma cells. The increased apoptosis seen was found to be the due partly to the inhibition of the NF-κB pathway.[62] In a SCID mouse model, Orr et al showed that NF-κB inhibition with liposome-encapsulated curcumin resulted in a significant decrease in disseminated neuroblastoma burden, with decreased NF-κB activity, decreased cellular proliferation, and increased apoptosis.[63]

Another transcription factor involved in neuroblastoma cell survival is MYCN, which is encoded by the human *MYCN* proto-oncogene. *MYCN* is part of a regulatory network controlling many aspects of cell function including cell proliferation and apoptosis.[64] As shown in a murine model, MYCN protein expression is usually restricted to early development and organogenesis. It is then severely downregulated within several days to weeks following birth and is usually only found in adult B cells.[65] When MYCN expression does not cease following birth, cell overgrowth and tumors result.[66] Mice genetically altered to express *MYCN*, had a significant incidence of developing neuroblastoma tumors. These tumors closely resembled those seen in humans.[67] Brodeur et al showed in 1984 that amplification of *MYCN* was found in neuroblastoma cell lines, and was associated with more aggressive, advanced tumor types. About half of unfavorable neuroblastomas were *MYCN* amplified and expressed high levels of MYCN, and *MYCN* amplification was associated with rapid tumor progression and worse outcomes.[23,68]

As a significant number of patients have *MYCN* amplified neuroblastoma, it is logical that anti-tumor therapies may target MYCN itself. For example, Burkhart and others showed that antisense oligonucleotides directed to human *MYCN* resulted in decreased MYCN protein expression and decreased cell proliferation in IMR-32 neuroblastoma cells. They also treated transgenic *MYCN* mice with antisense oligonucleotides directed at *MYCN* and showed an *in vivo* decrease in tumor growth.[69] Kang et al showed that MYCN protein expression could be significantly decreased using small interfering RNA against *MYCN* (si*MYCN*) in IMR-32 (*MYCN* amplified) neuroblastoma cells with no change in MYCN expression seen in SK-N-SH (*MYCN* non-amplified) cells. The silencing of *MYCN* inhibited cell growth and induced significant apoptosis after 48 hours of treatment in the *MYCN* amplified cell lines. Specifically, si*MYCN* was found to reduce expression of the anti-apoptotic protein Bcl-xL and increase caspase-3 mediated apoptosis. Other neuroblastoma cell lines (LAN-1, IMR-32, JF, SH-SY5Y, and SK-N-SH) were then treated with si*MYCN*, and significant apoptosis was found in each *MYCN* amplified cell line with no significant cell death in the non-amplified cell lines.[70] Similar results were seen with selective inhibition of *MYCN* using an anti-gene peptide nucleic acid (PNA). When IMR-32 (*MYCN* amplified) and SJ-N-KP (*MYCN* non-amplified) cells were treated with PNA, *MYCN* mRNA abundance decreased significantly, resulting in decreased cell growth and increased apoptosis that was more pronounced in the *MYCN*-amplified, IMR-32, cell line.[71] Since PNA form stable duplexes with DNA and RNA, and are resistant to nuclease and protease degradation, they show promise for potential antisense therapeutics against MYCN.[72] In a study examining phenotypic changes of *MYCN* amplified neuroblastoma cells, Lynch et al showed microRNA (miRNA) treatment of *MYCN* amplified cell lines with miR-335 resulted in decreased cellular migration and invasion.[73] Similarly, Buechner and colleagues utilized direct *MYCN*-targeting miRNAs, mir-101 and let-7e, in Kelly (*MYCN* amplified) neuroblastoma cells to block MYCN. They found a significant decrease in cellular proliferation when Kelly cells were treated with either of these two miRNAs.[74] Although amplification of the *MYCN* oncogene is associated with more aggressive tumor types, expression of MYCN protein does not always correlate with disease outcome.[75] For example, hyper-expression of MYCN protein in neuroblastoma cell lines with *MCYN* amplification, beyond that which is usually expressed, resulted in a significant reduction of neuroblastoma cell growth.[76] Finally, analyzing MYCN as a target for further drug

development, Lu et al developed a functional MYCN reporter gene assay using neuroblastoma cells stably transfected with a luciferase gene. This assay has been used to screen 2800 compounds from the Cancer Research-UK collection, identifying five compounds with significant reduction of MYCN-dependent luciferase activity (>50%). Further testing of these compounds is needed to determine the optimal method of MYCN inhibition.[77]

A third transcription factor found to play a role in cell survival in neuroblastoma is p53. This tumor suppressor gene is a regulator of the cell cycle that suppresses the growth of cancer cells, inhibits cell transformation, and induces apoptosis by inhibiting Bcl-2 protein and activating the pro-apoptotic protein BAX.[78] Activation of p53 is triggered by DNA damage[79] or hypoxia.[80] Mutations in p53 have been implicated in a plethora of cancers, to the extent that a 1994 database maintained by Hollstein et al found over 2500 somatic mutations in the p53 gene in various human tumors and tumor cell lines.[81]

In neuroblastoma, p53 mutations have been found to occur infrequently, and are often seen only in relapsing tumors.[82,83] Nonetheless, p53 plays an important role in these tumors through its influence upon other oncogenes. There are strong data to suggest that p53 and MYCN interact in neuroblastoma and may be mutually regulated in these tumors. Mutations of *p53* and amplification of *MYCN* have been shown to occur simultaneously in the same neuroblastoma tumors.[84] A study by Torres et al showed that co-transfection of the IMR5 neuroblastoma cell line with *p53* and *MYCN* resulted in elevated p53 expression and reduced MYCN expression.[76] Regan et al demonstrated that in *MYCN*-amplified neuroblastoma cell lines, an increase in p53 expression through inhibition of Hsp90, resulted in MYCN destabilization and suppression of cell growth.[85] Finally, employing chromatin immunoprecipitation assays and site directed mutagenesis p53 has been shown to be a direct transcriptional target of MYCN in neuroblastoma.[86]

Suppression of p53-dependent apoptosis by other proteins, such as MDM2[87,88], is one process cancer cells employ to avoid normal cell regulation. MDM2 is the primary negative regulator of p53 and has been found in neuroblastoma. Corvi et al showed that the *MDM2* gene was amplified in neuroblastoma cell lines, and also the MDM2 protein was present and bound to p53.[89] High frequency of single nucleotide polymorphisms in the *MDM2* gene promoter resulted in high levels of MDM2 expression that were associated with neuroblastoma disease aggressiveness.[90] Studies have shown that MDM2 is regulated by *MYCN* thereby regulating p53. Slack and colleagues, using chromatin immunoprecipitation, oligonucleotide pulldown and luciferase reporter assays demonstrated that MYCN binds to the *MDM2* promoter and induces MDM2 protein expression.[91] Conversely, MDM2 regulates MYCN expression. It was recently reported that MDM2 enhanced the mRNA and protein expression of MYCN at a post-transcriptional level. MYCN protein was reduced in *MYCN*-amplified neuroblastoma cell lines with silencing of MDM2 with siRNA.[92] In an *in vitro* study, He et al showed that in *MYCN* non-amplified neuroblastoma, MDM2 overexpression via gene transfection resulted in increased tumor growth and survival through suppression of p53. In *MYCN* amplified neuroblastoma, MDM2 served to induce MYCN expression, which was found to increase p53 expression as well. Subsequent inhibition of MDM2 via siRNA in these cells removed the p53 inhibition and resulted in decreased growth and induction of apoptosis due to the unrestricted p53 stimulation by MYCN itself.[93] Similar findings have been demonstrated *in vivo*. Utilizing murine models of human neuroblastoma xenografts, MDM2 knockdown with siRNA in cell lines expressing wild-type p53 resulted in decreased tumor growth and increased animal survival.[94]

Golubovskaya and others have recently demonstrated that p53 and FAK interacted in breast cancer cell lines[95] serving to sequester p53 in the cytoplasm and preventing its translocation to the nucleus.[96] They have also shown an interaction between MDM2 and FAK, and that interruption of the MDM2-FAK interaction with a small molecule resulted in decreased breast and colon tumor cell growth *in vitro* and *in vivo*.[97] Recent data in our laboratory indicate that p53 and FAK interact in neuroblastoma cell lines (Fig. 1) and that inhibition of the p53 and FAK interaction with interfering peptides results in decreased neuroblastoma tumor cell survival (Fig. 2). Therefore, targeting the inhibitors of p53 such as MDM2, or perhaps even better, the interactions between p53 and other proteins would be valid therapeutic strategies for neuroblastoma.

Neuroblastoma and Growth Factors

Growth factors also have an effect on cell survival. One of the better characterized growth factors functioning to promote cell survival is insulin-like growth factor (IGF), isoforms I (IGF-1) and II (IGF-II). Both type I and type II IGF receptors (IGF-IR, IGF-IIR) were present in neuroblastoma cells as shown by agonist binding studies, and IGF-1 and IGF-II stimulation both led to proliferation of the tumor cells, as Mattsson et al demonstrated in the SH-SY5Y cell line.[98] IGF-II has been detected in a little less than half of the neuroblastoma tumor specimens analyzed[99], and Tanno and colleagues found IGF-IR present in 86% of primary neuroblastoma tumor specimens studied.[100] Evidence also exists to support the idea that MYCN regulates the transcription of IGF-IR. When MYCN non-amplified neuroblastoma cells were transfected with MYCN cDNA, there was a marked increase in IGF-IR mRNA.[101] IGF stimulation caused neuroblastoma cells to initiate pro-survival pathways and avoid apoptosis. In 1996, Matthews and Feldman reported that administration of IGF-I to SH-SY5Y neuroblastoma cells during osmotic stress decreased apoptosis, and this effect was abrogated by a blocking antibody of IGF-IR.[102] In another study of hyperosmolar-induced apoptosis in neuroblastoma cells, Kim and colleagues demonstrated that IGF-1 exerted a protective effect against hyperosmolarity, decreasing apoptosis and promoting cell survival through activation of the PI3K/AKT pathway.[103] Another study showed IGF-1 protected SH-EP neuroblastoma cells from 1-methyl-4-phenylpyridinium (MPP+)-induced apoptosis, also via activation of the PI3K/AKT pathway.[104]

Insulin receptor substrate proteins 1 and 2 (IRS-1, IRS-2) are intracellular docking molecules that bind to phosphotyrosine residues on the IGF-1 receptor and mediate downstream transduction of the IGF signal.[105] The importance of IRS signaling in neuroblastoma survival has been noted. Kim and colleagues demonstrated that SH-EP neuroblastoma cells transfected with IRS-2 were resistant to hyperglycemia-induced apoptosis.[106] Similarly, it was shown that overexpression of IRS-1 or IRS-2 caused resistance to glucose-induced apoptosis via the PI3-kinase pathway.[107]

Some investigations evaluating IGF as a target for cancer therapies have been completed. NVP-AEW541 (Table 1), a small molecule inhibitor of IGF-IR activity, induced apoptosis in a number of neuroblastoma cell lines and also inhibited growth of HTLA-230 and SK-N-BE2(C) neuroblastoma xenografts in mice.[100] BMS-754807 (Table 1), an oral, reversible, ATP-competitive small molecule inhibitor of IGF-1R, was shown to inhibit the growth of neuroblastoma both *in vitro* and *in vivo;* however, response to the drug was defined as progressive disease with growth delay.[108] There are currently studies performing Phase I trials testing BMS-754807 in neuroblastoma, pending final results.[109] A recent study evaluating another IGF-1R inhibitor, MK0646 (Table 1), in metastatic neuroendocrine tumors, not including neuroblastoma, showed no partial or complete responses.[110] Therefore, single agent inhibition of the IGF pathway may not be beneficial, but its

inhibition in combination with other chemotherapeutic agents may be a target for future neuroblastoma therapies.

Epidermal growth factor (EGF) and the epidermal growth factor receptor (EGFR) is another pathway of growth factor signaling in neuroblastoma. EGFR expression has been demonstrated in neuroblastoma tumor specimens[111] and in a number of neuroblastoma cell lines.[112] The expression of EGFR was also found to be significantly increased in multi-drug resistant neuroblastoma cell lines both at the mRNA and protein level.[113] daMotta et al showed that EGFR ligand, EGF, treatment of SK-N-SH neuroblastoma cells resulted in cell proliferation.[114] Ho and colleagues confirmed these findings, but found that the EGF-stimulated proliferation was the result of EGFR activation of the PI3K/AKT pathway.[112] Tamura et al showed that gefitinib (Table 1), an EGFR tyrosine kinase inhibitor, induced apoptosis in neuroblastoma cells.[111] Hatziagapiou also illustrated that gefitinib treatment of SH-SY5Y neuroblastoma cells led to decreased tyrosine phosphorylation of EGFR and decreased cell survival.[115] Gefitinib was evaluated in a small study of children with relapsed neuroblastoma in combination with oral topotecan and cyclophosphamide, with encouraging results as measured as time to disease progression.[116] Other investigators have not shown such encouraging results. Rossler and others found that inhibition of cellular proliferation in neuroblastoma cells *in vitro* required concentrations of gefitinib that were not achievable in the clinical setting and felt that clinical trials were not warranted.[117] Another group looked at a small single-arm pilot study using gefitinib and irinotecan in 23 children with newly diagnosed neuroblastoma. They expected at least a 55% response rate, however, that level was not achieved in the 19 evaluable patients.[118] Another EGFR inhibitor, erlotinib (Table 1), has already completed Phase I clinical trials in children with a variety of refractory solid tumors including neuroblastoma, with few dose limiting toxicities and some long term responders.[119] Future research and clinical trials with EGFR inhibitors is warranted to develop a wider range of treatment options for neuroblastoma.

Platelet-derived growth factor (PDGF) is a platelet derived growth factor that influences neuroblastoma. PDGF is classically involved in the tissue healing processes as it stimulates cellular proliferation, chemotaxis, and matrix production[120], but the mitogenic pathway of PDGF is also involved in cellular transformation and malignancy in human tumors.[121] Pahlman et al demonstrated that a number of human neuroblastoma cell lines express PDGF receptor (PDGFR) but not PDGF. They also showed that PDGFR was functional in SH-SY5Y neuroblastoma cells in that PDGF stimulation resulted in a trophic and weakly mitogenic response.[122] The presence of PDGF receptor in neuroblastoma was also supported by work from Matsui et al. They showed PDGFR mRNA and protein expression in a variety of neuroblastoma cell lines. They proved functionality by stimulating neuroblastoma cell lines with ligands PDGF-A or PDGF-B and finding an increase not only in DNA incorporation, but also in cellular migration.[123] Eggert and others used RT-PCR to evaluate PDGF-A in human neuroblastoma specimens. They noted significantly more abundance of PDGF-A in advanced stage tumors (INSS Stage III and IV) compared to lower stage (I, II, IVS) tumors. In addition, they also found a positive correlation between PDGF-A mRNA and patient survival.[124] Further investigations have shown that the AKT pathway was responsible for the PDGF-stimulated proliferation migration, and invasion in neuroblastoma cells.[125]

Abrogating the effects of PDGF by inhibiting the PDGF receptor diminishes the ability of tumor cells to survive and propagate. Several PDGF receptor inhibitors have been shown to be effective in neuroblastoma. For example, multiple studies showed that gangliosides modify the effects of PDGF. In SH-SY5Y cells, gangliosides GM1, GM2, GT1b and GD1a were shown to inhibit the PDGF-stimulated phosphorylation of PDGFR and subsequent cellular proliferation.[126,127] Several drugs have been designed to inhibit the tyrosine kinase

activity of PDGFR. Imatinib (Table 1), a tyrosine kinase inhibitor of PDGFR, was shown to inhibit the growth of a number of human neuroblastoma cell lines *in vitro* and neuroblastoma xenografts *in vivo*[128], but a Phase II clinical trial of imatinib as a single agent failed to show a response in the treatment of recurrent or refractory neuroblastoma.[129] However, in a study published in 2009, the researchers showed that metronomic dosing of imatinib, in combination with doxorubicin, a common chemotherapeutic agent for neuroblastoma, resulted in cell cycle arrest and apoptosis in neuroblastoma lines *in vitro*. In addition, they showed significant decreases in tumor growth in established neuroblastoma xenografts.[130] A more recent study by Timeus et al investigated *in vitro* imatinib therapy in combination with the HIV protease inhibitor saquinavir. They discovered that the ability of saquinavir to inhibit cell proliferation and invasion as wells as induce apoptosis in neuroblastoma cell lines was significantly increased when combined with imatinib.[131] Other small molecule inhibitors of the PDGF pathway have been investigated. Backman et al tested a multi-targeted tyrosine kinase inhibitor directed at PDGFR, SU11657 (Table 1), in mice with SK-N-AS, IMR-32, and SH-SY5Y subcutaneous neuroblastoma xenografts. Treatment resulted in significant inhibition of tumor growth and decreased overall expression of PDGFR by immunohistochemistry.[132] A similar PDGFR pathway inhibitor, SU101 (Table 1), has been tested in children with refractory neuroblastoma and was well tolerated, although no clinical responses were documented.[133] This promising research highlights that PDGFR inhibition may be a prudent target for drug development for neuroblastoma.

Lastly, vascular endothelial growth factor (VEGF) has been shown to promote tumorigenesis in many human cancers including breast[134], non-small-cell lung[135], and prostate.[136] VEGF isoforms have been found in both neuroblastoma cell lines and in primary tumors[137], and the mRNA abundance[124,138] and protein expression were associated with higher stage tumors.[138,139] Vascular endothelial growth factor receptors (VEFGR) have been identified on neuroblastoma cells[140,141,142] as well as in primary tumor specimens.[143,138] Both VEGF and VEGFR are involved in promoting neuroblastoma cell survival. Treating IMR-32 neuroblastoma cells with VEGF protected them from apoptosis secondary to serum starvation or TNF-α.[144] In nude mouse neuroblastoma xenografts, inhibition of VEGF with anti-VEGF antibody resulted in significant decreases in xenograft growth.[145] VEGFR-2 blockade with gene silencing RNAi resulted in significant increases in apoptosis in serum starved neuroblastoma cells.[146] VEGF was especially significant in the subset of neuroblastomas with overexpression of *MYCN*. Kang et al found that *MYCN* inhibition with si*MYCN* significantly decreased VEGF secretion in *MYCN*-amplified neuroblastoma cells, and this effect was enhanced when combined with inhibition of PI3K/ AKT. These same effects were not observed in cells with low *MYCN*.[147] Inhibiting VEGF and VEGFR expression, therefore, should act on two fronts, reducing angiogenesis to the tumor as well as blocking cellular survival pathways.

Multiple inhibitors of VEGF and VEGFR have been recently developed. Bevacizumab, a humanized anti-VEGF-A antibody, reduced SK-N-AS, IMR-32, and SH-SY5Y neuroblastoma xenograft growth[148], and decreased tumor burden in animals with disseminated neuroblastoma.[149] In addition, administration of bevacizumab to neuroblastoma tumor bearing mice, prior to chemotherapy, improved the penetration of the chemotherapeutic drugs by 81%.[150] AZD2171 (Table 1), a relatively selective inhibitor of VEGFR, that is orally bioavailable, was shown to delay primary tumor growth in 5 of 6 neuroblastoma xenografts tested.[151] A Phase 1 study evaluating the safety of VEGF Trap administration to children with refractory solid tumors including neuroblastoma has been concluded with results pending publication. In a study by Rossler et al, inhibition of VEGFR in neuroblastoma xenografts with the pan-VEGFR tyrosine kinase inhibitor axitinib (AG-013736, Table 1) resulted in a significant delay in time needed to increase tumor

volume 5-fold and decreased microvessel density surrounding the tumors.[152] Similar results were seen by Orr et al in studying curcumin. They discovered that curcumin treatment decreased VEGF levels and microvessel density in neuroblastoma xenografts.[63] Another study utilized a small-molecule tyrosine kinase inhibitor, ZD6474 (vandetanib, Table 1), to block VEGFR-2. ZD6474 inhibited neuroblastoma cell viability *in vitro* and decreased neuroblastoma xenograft growth by 85%.[153] These studies show that targeting both the tumor cells and the tumor vasculature may be a more effective strategy to treat neuroblastoma.

In conclusion, cellular survival pathways are important for tumorigenesis in neuroblastomas. Patients with neuroblastoma continue to have poor prognosis, especially children with more aggressive tumors, such as those that have *MYCN* amplification. It is encouraging that many of the treatments targeting cell survival pathways produce a more profound effect in these aggressive cell lines. Continued research and discovery of effective inhibitors of these pathways will revolutionize the treatment of neuroblastoma.

Acknowledgments

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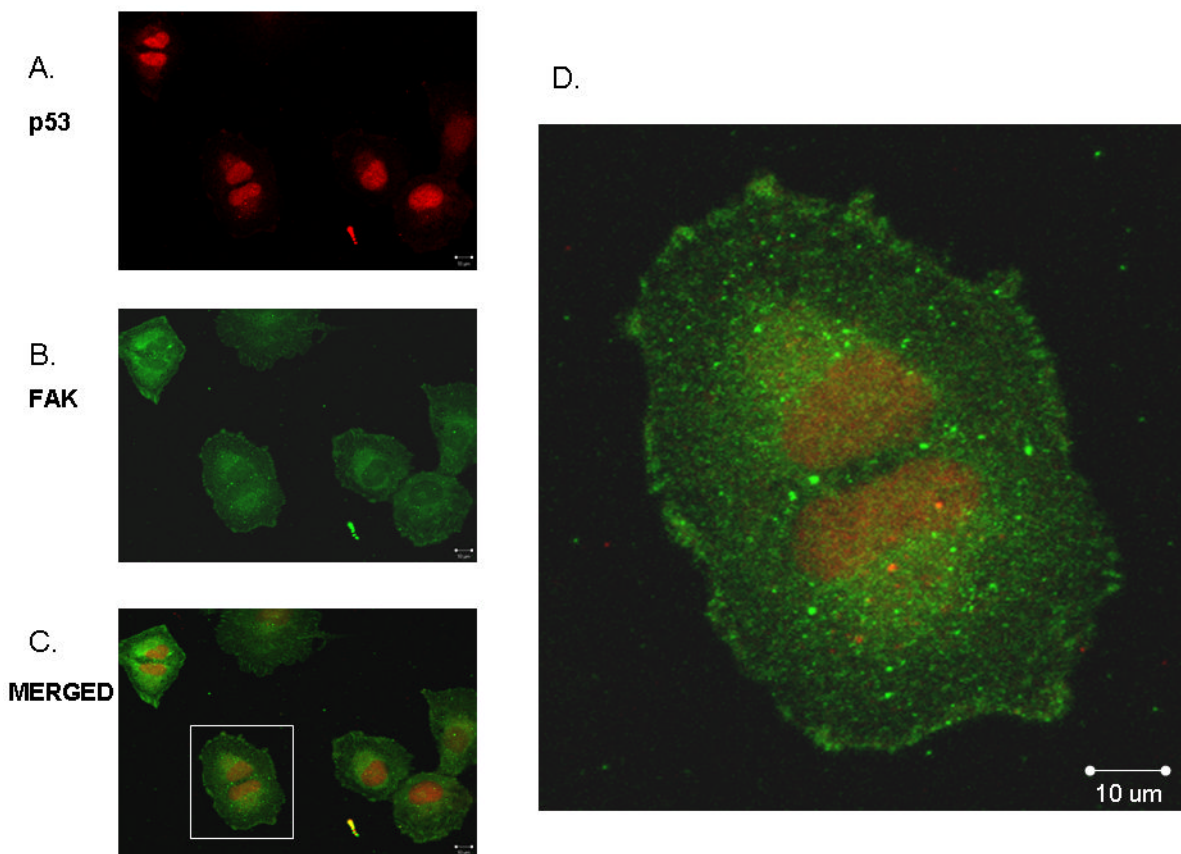
Figure 1.
Immunofluorescence staining followed by confocal microscopy was employed to evaluate p53 and FAK colocalization. SK-N-AS neuroblastoma cells were stained for p53 (red, A.) and FAK (green, B.) and evaluated with confocal microscopy to determine colocalization. Merged image (C) shows colocalization of the two stains with a Manders coefficient, [153] MA and MB of 0.68 and 0.33, respectively. D. Enlarged area of (C) (white box) to demonstrate colocalization of the two stains in the nucleus and perinuclear areas.
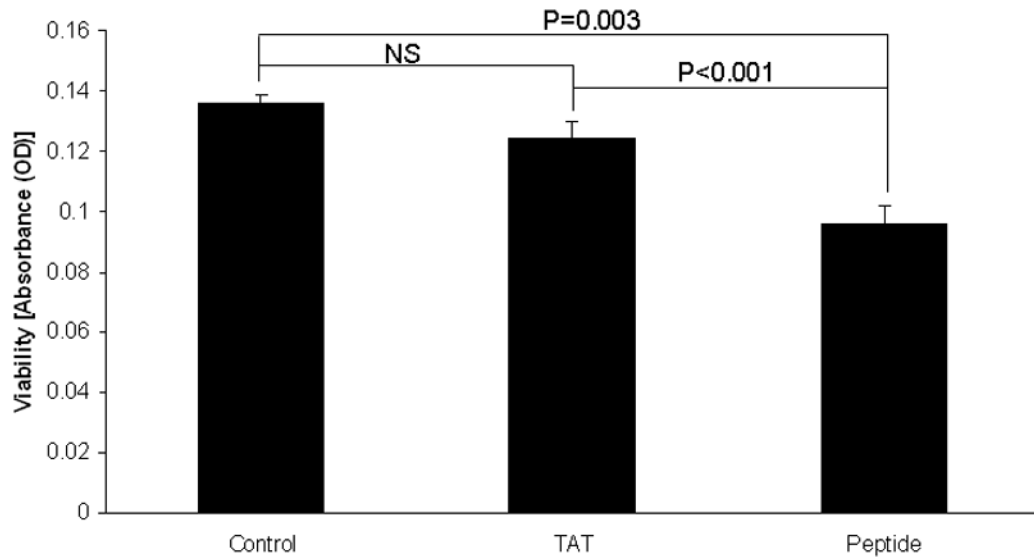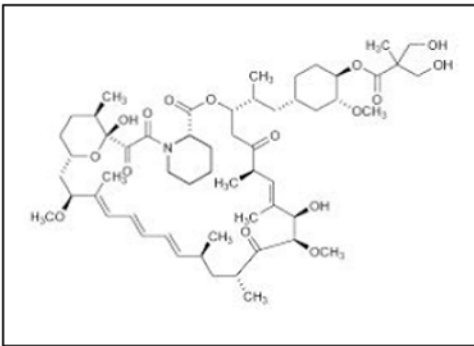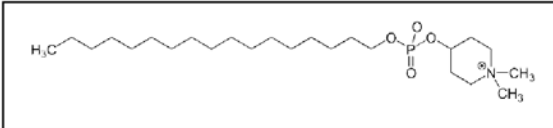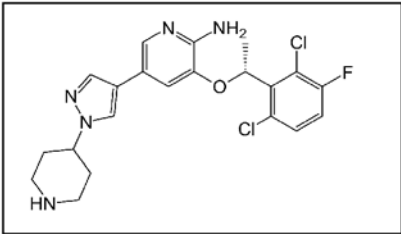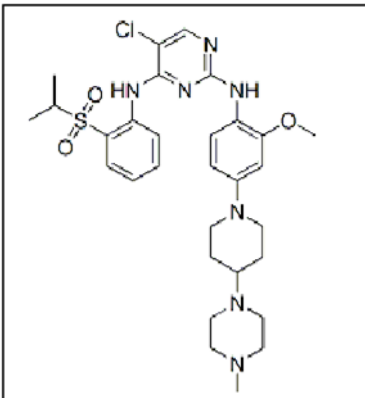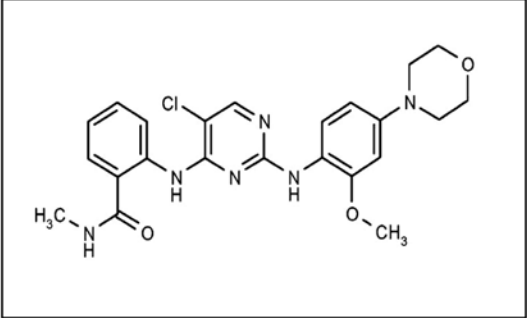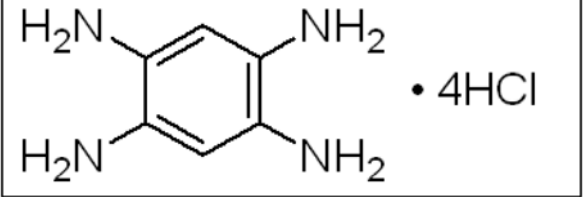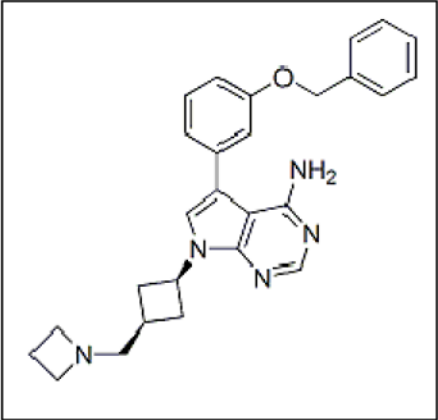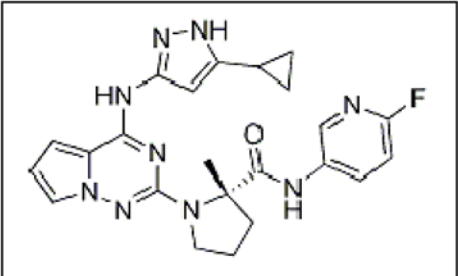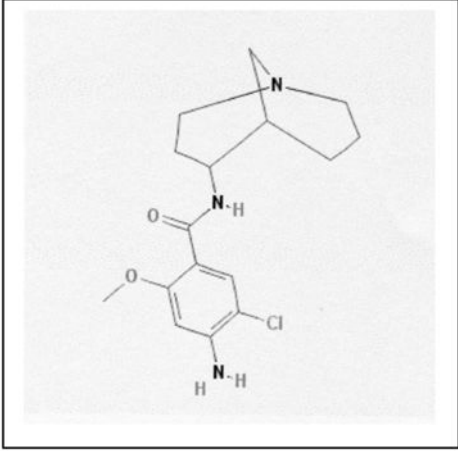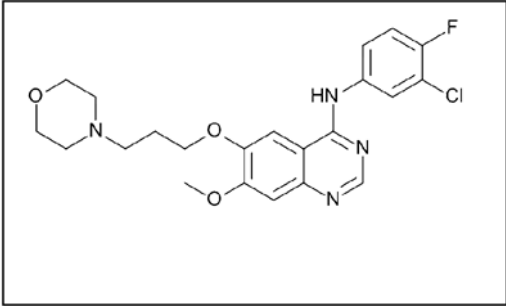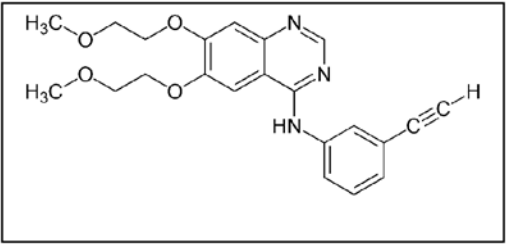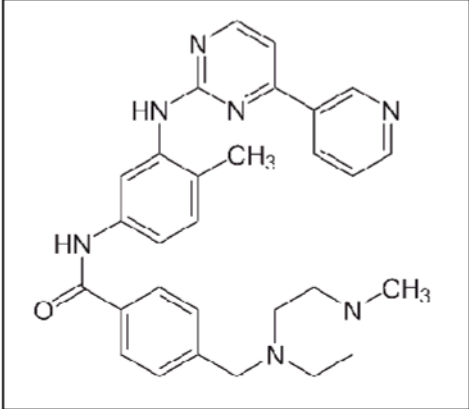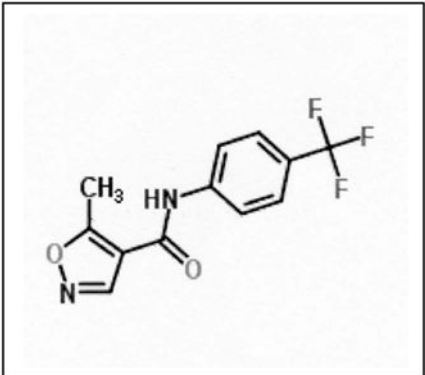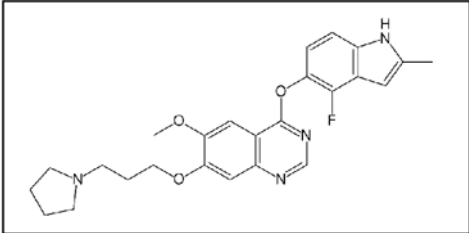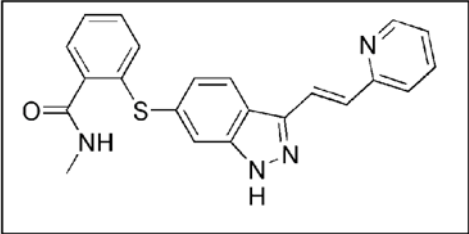
Figure 2.
SH-EP neuroblastoma cells were treated with a 7-mer peptide to disrupt the p53-FAK
interaction and compared to untreated cells and cells treated with the TAT sequence alone.
Cell viability was measured with alamarBlue® assay. There was a significant decrease in
cell viability following peptide treatment compared to either control cells or cells treated
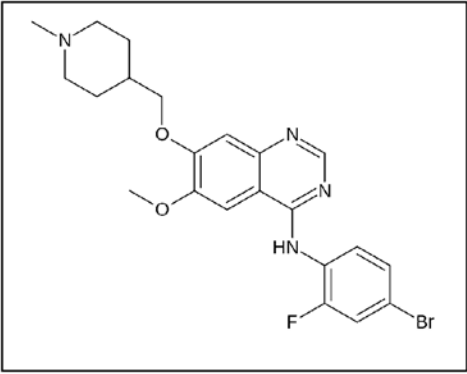with TAT sequence alone. The TAT sequence did not significantly affect cell viability.

Table 1

Inhibitor Name	Chemical Name	Structure	Company
Temsirolimus	(1R,2R,4S)-4-{(2R)-2-[(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,27-dihydroxy-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-1,5,11,28,29-pentaoxo-1,4,5,6,9,10,11,12,13,14,21,22,23,24,25,26,27,28,29,31,32,33,34,34a-tetracosahydro-3H-23,27-epoxypyrido[2,1-c][1,4]oxazacyclohentriacontin-3-yl]propyl}-2-methoxycyclohexyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate		Wyeth
Perifosine	1,1-Dimethylpiperidinium-4-yl octadecyl phosphate		Keryx
Crizotinib	3-[(1R)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridin-2-amine		Pfizer
NVP-TAE684	5-chloro-N4-(2-(isopropylsulfonyl)phenyl)-N2-(2-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)pyrimidine-2,4-diamine		Novartis

Inhibitor Name	Chemical Name	Structure	Company
NVP-TAE226	(2-[5-Chloro-2-[2-methoxy-4-(4-morpholinyl)phenylamino]pyrimidin-4-ylamino]-N-methylbenzamide		Novartis
Y15	1,2,4,5-Benzenetetraamine tetrahydrochloride		Sigma
NVP-AEW541	7-((1s,3s)-3-(azetidin-1-ylmethyl)cyclobutyl)-5-(3-(benzyloxy)phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine		Novartis
BMS-754807	(S)-1-(4-((5-cyclopropyl-1H-pyrazol-3-yl)amino)pyrrolo[2,1-f][1,2,4]triazin-2-yl)-N-(6-fluoropyridin-3-yl)-2-methylpyrrolidine-2-carboxamide		Bristol-Myers Squi

Inhibitor Name	Chemical Name	Structure	Company
MK0646	4-amino-N-(1-azabicyclo[3.3.1]nonan-4-yl)-5-chloro-2-methoxybenzamide		Merck
Gefitinib	*N*-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine		AstraZeneca
Erlotinib	*N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy) quinazolin-4-amine		Genentech

Inhibitor Name	Chemical Name	Structure	Company
Imatinib	4-[(4-methylpiperazin-1-yl)methyl]-*N*-(4-methyl-3-{[4-(pyridin-3-yl)pyrimidin-2-yl]amino}phenyl)benzamide		Novartis
SU11657	Unknown	Unknown	Sugen
SU101	5-Methyl-N-[4-(trifluoromethyl)phenyl]-1,2-oxazole-4-carboxamide		Sugen
AZD2171	4-[(4-fluoro-2-methyl-1*H*-indol-5-yl)oxy]-6-methoxy-7-[3-(pyrrolidin-1-yl)propoxy]quinazoline		AstraZeneca
Axitinib	*N*-Methyl-2-[[3-[(*E*)-2-pyridin-2-ylethenyl]-1*H*-indazol-6-yl]sulfanyl]benzamide		Pfizer

Inhibitor Name	Chemical Name	Structure	Company
Vandetanib	*N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine		AstraZeneca

This table provides a list of the chemical names, structures and companies for the inhibitors discussed in the manuscript.