ELMER PRESS

Original Article

World J Oncol. 2024;15(3):492-505

Synergistic Effects of Neratinib in Combination With Palbociclib or Miransertib in Brain Cancer Cells

Ermira Mulliqi[a], Said Khelwatty[a], Anna Morgan[a], Keyoumars Ashkan[b], Helmout Modjtahedi[a, c]

Abstract

Background: Aberrant expression and activation of epidermal growth factor receptor (EGFR) resulted in approval of several forms of EGFR inhibitors in the treatment of patients with a wide range of epithelial cancers. However, no EGFR inhibitor has yet been approved for the treatment of patients with brain cancer, indicating that targeting EGFR alone may not be sufficient in some patients.

Methods: In this study, we investigated the role of all members of the EGFR family, other growth factor receptors, cell-cycle proteins, and downstream cell signaling pathways (e.g., mitogen-activated protein kinase (MAPK), serine/threonine protein kinase (AKT), signal transducer and activator of transcription (STAT3), Src, Abelson murine leukemia viral oncogene homolog (Abl)) on the growth of a panel of human brain cancer cell lines (HBCCLs). We examined the growth response of HBCCLs to treatment with 17 targeted agents compared to two cytotoxic drugs.

Results: Of the targeted agents, the irreversible pan-human epidermal growth factor receptor (HER) inhibitors neratinib and afatinib were more effective than erlotinib and lapatinib at inhibiting the growth of all HBCCLs, and the cyclin-dependent kinase (CDK)1/2/5/9 inhibitor dinaciclib was the most potent targeted agent. We found that treatment with Src/Abl/c-kit inhibitor dasatinib, signal transducer and activator of transcription (STAT3) inhibitor stattic, Abl/platelet-derived growth factor receptor (PDGFR)α/vascular endothelial growth factor (VEGFR)2/fibroblast growth factor receptor (FGFR)1 inhibitor ponatinib, and the tropomyosin receptor kinase (TRK)/ROS proto-oncogene 1 receptor tyrosine kinase (ROS)/anaplastic lymphoma kinase (ALK) inhibitor entrectinib, also inhibited the growth of all HBCCLs. Interestingly, these agents were more effective in inhibiting growth of HBCCLs when proliferating at a slower rate. In addition to inhibiting the proliferation of HBCCLs, treatment with neratinib, dinaciclib, dasatinib, stattic and trametinib inhibited the migration of brain tumor cell line A172.

Conclusions: Notably, we found that treatment with neratinib in combination with palbociclib (CDK4/6 inhibitor), or miransertib (AKT1/2/3 inhibitor) resulted in synergistic growth inhibition of all HBCCLs. Our results support that repurposing drugs like neratinib in combination with the palbociclib or miransertib may be of therapeutic potential in brain cancer and warrants further investigations.

Keywords: Brain cancer; EGFR; Cancer stem cell markers; Neratinib; Dasatinib; Palbociclib; Miransertib

Introduction

Brain cancer is one of the deadliest and most lethal types of human cancers. Glioblastoma (GB) is one of the most frequent primary brain tumors in adults with an extremely poor prognosis, with a median overall survival (OS) and a 5-year OS of 8 months and 7%, respectively [1]. At present, standard treatment for brain cancer patients involves surgery based on the anatomical location of the tumor and neoadjuvant chemotherapy [2, 3]. However, brain cancer cases are diagnosed in patients at an advanced stage of the disease, and most patients have poor response to the currently available therapeutic interventions [3, 4]. As a result, there is an urgent need for identification of reliable biomarkers for the early detection of brain cancer, as well as therapeutic targets and the development of more effective therapeutic interventions for such patients [5].

Since the early 1980s, aberrant expression and activation of the human epidermal growth factor receptor (EGFR) has been reported in a wide range of human malignancies. The epidermal growth factor RTK family consists of four members: EGFR (ErbB1, human epidermal growth factor receptor (HER)1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [6, 7]. EGFR ligands are synthesized as type 1 transmembrane precursors that undergo extracellular domain cleavage to release soluble ligands, which then bind to and activate the EGFR [8]. The seven ligands include epidermal growth factor (EGF), transforming growth factor (TGF)α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin (BTC), amphiregulin (AREG) and epithelial mitogen (EPGN). The binding of ligands to the extracellular domain of the EGFR results in the formation of EGFR homodimerization or its het-

Manuscript submitted March 19, 2024, accepted April 26, 2024
Published online May 7, 2024

[a]School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Kingston, UK
[b]King's College Hospital, NHS Foundation Trust, London, UK
[c]Corresponding Author: Helmout Modjtahedi, School of Life Science, Pharmacy and Chemistry, Faculty of Health, Science, Social Care and Education, Kingston University London, Kingston-Upon-Thames, Surrey KT1 2EE, UK. Email: H.Modjtahedi@Kingston.ac.uk

doi: https://doi.org/10.14740/wjon1873

erodimerization with the other three members of the EGFR family, which include HER-2 (Neu, c-ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). As a result, an auto and/or transphosphorylation of C-terminal region of the intracellular tyrosine kinase domain leads to the activation of various downstream constituents of signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)/serine/threonine protein kinase (AKT), RAS/RAF/mitogen-activated protein kinase (MAPK), Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and PLC-γ1. This activation, in turn, contributes to the set of the most prominent hallmarks of a cancer including tumor cell proliferation, migration, and invasion [9]. To date, several inhibitors targeting one or more members of the HER family have been approved for the treatment of patients with a wide range of human cancers but not yet in patients with brain cancer [6, 10].

In several studies, increased expression, activation and crosstalk between the HER family members, increased expression of other growth factor receptor systems and the presence of brain cancer stem cells (BCSCs) have been associated with poor response to treatment with the HER inhibitors and poor prognosis [11-13]. To date temozolomide (TMZ) is the only drug that has gained the Food and Drug Administration (FDA) approval for treatment for patients with brain cancer, but it is accompanied by toxicity in some patients, highlighting the importance of discovering more specific therapeutic targets. In addition, due to the heterogenous nature of brain tumors, it is essential to investigate the relative expression of all members of HER family, and other biomarkers in the progression of brain cancer [14]. Furthermore, it is also important to determine the responses of brain tumor cells to the treatment with various types of HER inhibitors when used alone or in combination with other agents targeting different cell signaling molecules and pathways [15, 16].

As a result, in this study, we investigated the role of all members of the HER family, other growth factor receptors (e.g., hepatocyte growth factor receptor (c-MET)), cell-cycle proteins, the putative BCSC biomarker CD44 and downstream cell signaling pathways in the growth of a panel of human brain cancer cell lines (HBCCLs). Moreover, the therapeutic potential of various agents targeting such proteins when used alone or in combination on the growth of a panel of HBCCLs, established from patients with GB, were also investigated.

Materials and Methods

Tumor cell lines

A panel of four human brain cancer cell lines (HBCC) were examined in this study. Of these LN-18, U118MG and T-98G were purchased from the American Tissue Culture Collection (ATCC, LGC, Teddington, UK), and A172 from the European Collection of Authenticated Cell Cultures (ECACC, UK health security agency, Sailsbury, UK). All cell lines were cultured routinely at 37 °C in a humidified atmosphere (5% CO_2). A172, U118MG and T-98G were cultured in Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich, Dorset UK), containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK) and antibiotics penicillin (50 U/mL), streptomycin (50 µg/mL) and neomycin (50 µg/mL). LN-18 was also cultured in DMEM as above but in 5% FBS, and T-98G was supplemented with 100 mM pyruvate (Gibco, UK).

Tyrosine kinase inhibitors (TKIs) and antibodies

Erlotinib, lapatinib, neratinib, afatinib, palbociclib, ribociclib, dinaciclib, capmatinib, dasatinib, stattic, ponatinib, entrectinib, AZD4547, trametinib, selumetinib, miransertib, lorlatinib, docetaxel and paclitaxel were all purchased from Selleckchem (Suffolk, UK) and made at stock concentration of 10 mM and solubilized in dimethyl sulfoxide (DMSO) as instructed on the data sheet. The antibodies for flow cytometry included our inhouse mouse anti-EGFR (HM43.16B) and anti HER2 (HM50.67A) antibodies. The mouse monoclonal antibodies against HER3 (MAB3481), HER4 (MAB11311), ALK7 (MAB77491) and HGF R/c-MET (MAB3582) were purchased from R&D Systems (Oxford, UK). The mouse anti-CD44 antibodies (immunoglobulin (Ig)G2b clone G44-26/27) were supplied by Becton Dickinson (Oxford, UK). The anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibodies STAR9B was purchased from Serotec Ltd. (Oxford, UK). All purchased and inhouse antibodies were solubilized in sterile phosphate buffered saline (PBS) and stored at 4 °C in the fridge for short-term use, at stock concentrations of 1 mg/mL for inhouse antibodies, and 0.5 mg/mL for purchased antibodies. Antibodies used in Western blot included the mouse anti-EGFR antibody (clone F4) (E3138-2ml), which was obtained from Sigma-Aldrich, Merck KGaA (Dorset, UK). Other antibodies for the Western blot analysis, such as the rabbit anti-phospho-EGFR (Tyr1068), HER2 (2242s), phosphor-HER2 (Tyr1221/1222) (2243), phosphor-HER3 (Tyr1289) (4791), phosphor-HER4 (Tyr1284)/EGFR (Tyr1173) (4757), phosphor-MAPK (Tyr202/Tyr204) (4370), phospho-Akt (S473) (4060), phospho-STAT3 (Y705) (9145), phospho-SRC (Y416) (Tyr416) (6942) and β-actin (4970), were all obtained from Cell Signaling Technology Inc. (Hitchin, UK). Both the goat anti-mouse IgG IRDye 800CW and donkey anti-rabbit IgG IRDye 680RD were purchased from LI-COR Ltd., solubilized in sterile PBS and stored at 4 °C (Cambridge, UK). All Western blot antibodies were purchased in solubilized conditions and stored at -20 °C.

Flow cytometry

The expression of various markers in the brain cancer cell lines was accessed by flow cytometry as described previously [17]. The cells (approximately 5×10^5) suspended in 2% FBS medium were added to 1.5 mL Eppendorf, centrifuged (254 × g for 3 min), washed once with cold PBS and incubated with or without 10 µg/mL of the primary antibody by rotation at 4 °C for 1 h. Following that, the cells were washed thrice with 1 mL of cold PBS by centrifugation (254 × g for 3 min) and incubated with secondary antibody STAR9B (1:200 dilution) by rotation at 4 °C for 1 h. Finally, the cells were washed thrice with cold

PBS by centrifugation and resuspended in 1 mL of PBS. The fluorescence-activated cell sorting (FACS) analysis was carried out using Guava EasyCyteTM flow cytometry (Luminex Corp). A minimum of 10,000 events were measured through excitation of argon laser using Green-B fluorescence (525/30 nm) and analyzed using IncyteTM soft 3.3 (Luminex Corp.).

Tumor growth response studies

The effect of various agents on the *in vitro* growth of tumor cells was determined using sulforhodamine B (SRB; Sigma-Aldrich; Merck KgaA, Dorset, UK) colorimetric assay as previously discussed by [17]. Briefly, 5,000 cells in 100 µL were seeded per well of a 96-well plate in medium containing 10% or 2% FBS and incubated at 37 °C (in a humidified atmosphere in 5% CO_2). Following a 4-h incubation, "time zero" plate (representing the initial number of cells prior to treatment) was fixed with 10% trichloroacetic acid (Fisher Scientific, Loughborough, UK) for 1 h at room temperature, washed thrice with tap water and left to air dry overnight. For other plates, 100 µL of doubling dilutions of agents were added to each well in triplicate and incubated at 37 °C until the controls (medium only) became confluent. These plates were then fixed as mentioned above, stained with 0.04% (w/v) SRB in 1% acetic acid for 1 h, washed thoroughly with 1% acetic acid and left to air dry overnight. The stained cells were solubilized with 100 µL/well of 10 mM Tris-base (pH 10), and the absorbance of each well was measured at 565 nm using an Epoch plate reader (Thermo Fisher Scientific, Inc.). Growth as a percentage of control was determined using the following formula:

$$\% \text{ Cell Growth} \frac{X-Y}{Z-Y} \times 100$$

whereas X is the absorbance of the drug-treated well at 565 nm, Y is the absorbance prior to treatment at 565 nm, and Z is the absorbance of the untreated cells at 565 nm. The 50% inhibitory concentration of each agent (IC_{50}) was calculated using the non-linear least squares curve fitting (four parameter analysis, log (inhibitor) vs. response, variable slope) using Gen5 software (BioTeck, UK).

Determination of combination index

The effect of selected agents on the growth of HBCCLs when used in combination was assessed using SRB assay as described previously [17]. For each combination, two agents (TKIs or cytotoxic agent) were mixed at their respective 4 × IC_{50} value (determined previously as a single agent) followed by eight doubling dilutions. Data analysis was performed using Calcusyn software (Biosoft, UK) and interpreted as follow: < 0.9 = synergistic effect, 0.9 - 1.1 = additive effect, > 1.1 = antagonistic effect.

Cell cycle distribution analysis

The effect of a range of selected agents including HER family members, cyclin-dependent kinase (CDK), Src, STAT3 and cytotoxic agent on the cell cycle distribution of HBCCLs was investigated using flow cytometry. Approximately, 0.5 × 10^6 cells/well were seeded in six-well plate containing 5 mL of 10% FBS medium with or without drugs at IC_{70} and incubated at 37 °C until the control wells (no drugs) became almost confluent. Following that, the cells were harvested by trypsinization and pooled with their respective supernatants, washed once with cold PBS by centrifugation (264 × g for 4 min) and fixed with 70% ice-cold ethanol for minimum of 3 h at -20 °C. The cells were collected by centrifugation (450 × g for 5 min) washed thrice with cold PBS and stained with Guava cell cycle reagent (Luminex Corp, USA). Cells were then run through a Guava EasyCyte™ flow cytometer (Luminex Corp.), where 10,000 events were recorded by excitation with an argon laser (488 nm) using Yellow-B fluorescence (583/26 nm) and analyzed using Incyte™ soft 3.3 (Luminex Corp).

Western blot analysis

Effectiveness of various agents on the downstream signaling molecules of A172 cell line was investigated using Western blot analysis. Briefly, 0.5 × 10^6 cells/well were grown in 5 mL of 10% FBS DMEM medium in six-well plate to near confluency. Cells were washed once with 5 mL of 0.5% FBS DMEM medium and incubated at 37 °C with the desired drug at a final concentration of 400 nM (or no inhibitor/medium only as a negative control) in 5 mL of fresh 0.5% FBS DMEM medium for 1 h. After that, A172 was incubated for a further 15 min with 30 nM of EGF, HB-EGF or no ligand. The cells were then washed once with PBS and lysed with 300 µL of preheated lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) containing protease inhibitor cocktail (Sigma-Aldrich; Merck KgaA) and homogenized using 25 × 5/8′ gauge needles to reduce its viscosity. Protein samples (25 µg) were separated on 4-12% Bis-Tris gel (Invitrogen; Thermo Fisher Scientific, Inc.) using the Xcell II Surelock Mini-Cell system (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto immobilon-polyvinylidene fluoride (FL PVDF) membranes (Merck), using Xcell II Mini-Cell Blot Module kit (Invitrogen; Thermo Fisher Scientific, Inc.). The immobilon PVDF transfer membranes were probed with various antibodies at the manufacturer's recommended dilutions and visualized using the LI-COR Image Studio software.

Migration assay

The effect of selected agents on the migration of HBCCLs was investigated using chemotaxis. The cell migration assay was conducted using the IncuCyte Clear View 96-well cell migration plate according to the manufacturer's instructions (Essen Bioscience Ltd. Hertfordshire, UK) as described previously [18]. Approximately 25 × 10^3/mL tumor cells plus the treatment in total volume of 60 µL 0.5% FBS medium were added into Clear View 96-well insert [18]. Each cell plate was then left to settle at the room temperature for 15 min followed by in-

Table 1. Surface Expression of Various Growth Factor Receptors in Human Brain Cancer Cell Line (Determined by Flow Cytometry)

Cell line	Mean fluorescence intensity (MFI)							
	Control	EGFR	HER2	HER3	HER4	C-MET	ALK-7	CD44
LN-18	3.7	32.9	8.8	4.2	3.9	5.8	4.4	3.9
U118MG	3.5	28.5	5.8	3.9	4.0	5.4	3.9	1,354.6
A172	3.0	43.6	9.4	3.5	3.4	7.7	3.8	1,439.0
T-98G	3.2	33.8	8.9	3.5	3.6	5.9	3.6	3,259.8
HN5	2.9	1071.7	N/A	N/A	N/A	N/A	N/A	N/A
SKOV3	3.8	N/A	233.3	N/A	N/A	N/A	N/A	N/A
CaCo2	2.4	N/A	N/A	N/A	N/A	N/A	N/A	49.51

The data are presented as the mean fluorescence intensity (MFI) ± standard deviation (SD) of gated events. N/A: not available. ALK anaplastic lymphoma kinase; c-MET: hepatocyte growth factor receptor; CD: cluster differentiation; EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor.

cubation for a further 30 min at 37 °C. Then 200 µL of medium containing 10% FBS (chemoattractant) was added to the lower chamber. The cell plate was then placed onto the IncuCyte Zoom® instrument and was left for 15 min at 37 °C to settle. After removal of any condensation on the lid, the plate was returned to the IncuCyte Zoom instrument with a 10 × objective using the IncuCyte™ chemotaxis system. Chamber wells were analyzed every 3 h using the IncuCyte chemotaxis software.

Statistical analysis

The statistical analysis was carried out using SPSS software (IBM®, SPSS statistics version 26) as described previously [18]. Linear regression analysis was used to assess the relationship between the expression of HER family members and response to treatment with various TKIs, CDK inhibitor, STAT3 inhibitor and cytotoxic agent. The effect of selected agents on the migration of brain cancer cell lines were tested by paired *t*-test analysis. A P value of ≤ 0.05 was statistically significant, and an R^2 value closer to 1 showed the reliability of the association between the IC_{50} value of each drug and expression level of each marker.

Ethics compliance

The study did not involve the use of patient samples, therefore Institutional Review Board (IRB) approval and ethical compliance were not applicable.

Results

Cell surface expression of various growth factor receptors and CD44 in brain cancer cell lines

We determined the expression levels of all members of the HER family, c-MET, ALK7 and CD44 in HBCCLs by flow cytometry where results are represented in Table 1 and Figure 1. Most of the HBCCLs had moderate expression of EGFR and little to no expression of HER2, HER3 and HER4. In comparison to the overexpressing EGFR control head and neck cancer cell line HN5 (mean fluorescence intensity (MFI) = 1,071.7), the highest level of EGFR expression was found in A172 cells (MFI = 44). The expression level for HER2, HER3 and HER4 was very low in all cell lines and almost undetectable for some. Similarly, the expression levels of c-MET and ALK7 were also very low in all HBCCLs. Finally, with the exception of LN-18, which was found to be CD44 negative, all the remaining three brain cancer cell lines had overexpression of CD44, with MFI values ranging from 1,355 (U118MG) to 3,260 (T98-G).

Growth response of human brain cancer cell lines to treatment with various targeted agents

Using the SRB assay, we investigated the effect of various agents on the growth HBCCLs and the impact on tumor cell proliferation rate on the IC_{50} value of each drug when cultured in medium containing 10% FBS (5 - 6 days to reach confluency) and 2% FBS (8 - 9 days to reach confluency). The effects of these agents on the growth of HBCCLs when cultured in medium containing 2% and 10% FBS are presented in Table 2. From the four different types of the HER TKIs targeting one or more of the HER family members in medium containing 2% FBS, the irreversible pan-HER family blocker neratinib was most effective by inhibiting the growth of all four brain cancer cell lines, with IC_{50} ranging from 0.30 to 0.69 µM (Table 2, Fig. 2). This was followed by the CDK1/2/5/9 inhibitor dinaciclib which inhibited the growth of all four cell lines with the IC_{50} values ≤ 0.16 µM. Of the other targeted agents, the Abelson murine leukemia viral oncogene homolog (Abl)/Src/c-Kit inhibitor dasatinib inhibited the growth of HBCCLs with IC_{50} values of 0.01 µM (U118MG) to 0.04 µM (A172), the STAT3 inhibitor stattic with IC_{50} values of 0.61 µM (A172) to 9.68 µM (T-98G). In addition, treatment with the Abl/platelet-derived growth factor receptor (PDGFR)α/vascular endothelial growth factor (VEGFR)2/fibroblast growth factor receptor (FGFR)1 inhibitor ponatinib inhibited HBC-

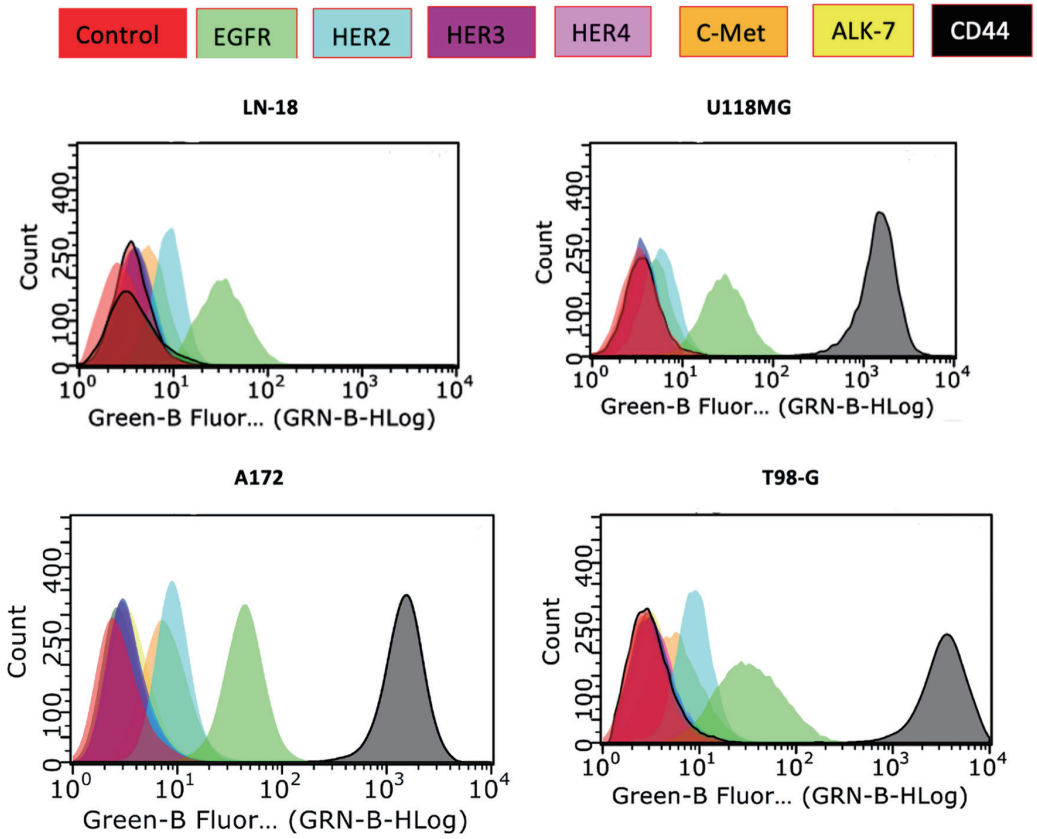

Figure 1. The expression levels of various growth factor receptors in human brain cancer cell lines determined by flow cytometry and represented as histograms. EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor; c-MET: mesenchymal-epithelial transition factor; ALK7: anaplastic lymphoma kinase 7: CD44: cluster differentiation 44.

CLs growth with IC_{50} values ranging from 0.03 µM (A172) to 0.40 µM (U118MG), and the TrkA/B/C/ROS/ALK inhibitor entrectinib with values ranging from 0.11 µM (LN-18) to 1.19 µM (U118MG), respectively. In contrast, the AKT1/2/3 inhibitor miransertib had moderate effect on growth of all HBCCLs, treatment with c-MET capmatinib, MEK/ERK1/ERK2 inhibitor selumetinib and ALK/Ros1 inhibitor lorlatinib had minimum to no inhibitory effect on the growth of all four HBCCLs (Table 2).

Cell cycle distribution analysis

The effect of various agents on the cell cycle distribution of all four HBCCL's was determined using flow cytometry, and results are presented in Table 3. Treatment for all four brain tumor cell lines with dinaciclib, dasatinib, stattic and paclitaxel was accompanied by upregulation of cells in subG1 phase of the cell cycle. In contrast, treatment with neratinib resulted in upregulation of cells in the subG1 phase in two of the four brain cancer lines examined (A172 and LN-18). In addition, whereas treatment with all agents resulted in upregulation of the cells in the S phase of cell cycle in A172, the same treatments reduced the percentage of cells in the S phase in U118MG cells, highlighting the heterogenous nature of brain cancer cells.

Treatment with neratinib and miransertib blocks phosphorylation of EGFR, MAPK and AKT

The effects of treatment with neratinib, dinaciclib, dasatinib, stattic and miransertib on the EGF- and HB-EGF-induced phosphorylation of growth factor receptors and downstream cell signaling molecules were examined in A172 cells, and the results are presented in Figure 3. In common to FACS analysis, the expression of phosphorylated HER2, HER3 and HER4 were undetectable in A172 cells (data not shown). However, treatment with neratinib blocked the phosphorylation of EGFR at position 1,068, which resulted in reduction in the phosphorylation of downstream signaling molecules such as MAPK and AKT in A172 cell line. Moreover, treatment with miransertib also resulted in reduced EGF- or HB-EGF-induced phosphorylation of EGFR, MAPK and AKT (Fig. 3).

Effect of selected agents on the migration of brain cancer cell lines

Increased migration is another hallmark of human cancers. Next, we examined the effect of various targeted agents on the migration of human brain cancer cell line A172, using In-

Table 2. IC_{50} Values of Various Agents on HBCCLs as Assessed by SRB Colorimetric Assay: (A) HER-Family Targeting TKIs and Other Downstream Signaling Molecules and (B) Other TKIs and Chemotherapeutic Agents

IC_{50} value (μM)	% FBS	LN-18	A172	U118MG	T-98G
A					
Erlotinib (EGFR inhibitor)	2%	6.42	10.00	7.45	6.84
	10%	> 10.00	> 10.00	> 10.00	> 10.00
Lapatinib (EGFR/HER2 inhibitor)	2%	1.08	10.00	5.60	3.25
	10%	8.55	10.00	6.39	10.00
Neratinib (EGFR/HER2/HER4 inhibitor)	2%	0.30	0.35	0.33	0.69
	10%	1.55	1.12	0.44	1.94
Afatinib (EGFR/HER2/HER4 inhibitor)	2%	1.11	1.24	1.49	1.85
	10%	2.97	2.50	1.42	4.04
Palbociclib (CDK4/CDK6 inhibitor)	2%	1.94	1.16	0.45	0.56
	10%	2.07	4.69	2.46	4.78
Dinaciclib (CDK1/CDK2/ CDK5/CDK9)	2%	0.006	0.004	0.014	0.013
	10%	0.008	0.003	0.0125	0.010
Ribociclib (CDK4/CDK6 inhibitor)	2%	1.62	5.76	1.43	5.50
	10%	5.80	4.13	1.00	> 10.00
Capmatinib (C-MET inhibitor)	2%	> 10.00	> 10.00	4.22	> 10.00
	10%	> 10.00	> 10.00	> 10.00	> 10.00
Dasatinib (Abl/Src/c-Kit)	2%	0.041	0.04	0.01	0.01
	10%	1.80	2.96	0.10	0.06
Stattic (STAT3 inhibitor)	2%	1.21	0.61	1.13	9.68
	10%	3.76	0.72	1.00	> 10.00
B					
Ponatinib (Abl/ PDGFRα/VEGFR2/FGFR1 inhibitor)	2%	0.10	0.03	0.40	0.19
	10%	0.41	0.19	0.88	0.42
Entrectinib (TrkA/B/C/ROS/ALK inhibitor)	2%	0.11	0.78	1.19	0.95
	10%	2.95	2.85	2.93	3.47
AZD4547 (FGFR 1/2/3 inhibitor)	2%	1.61	0.01	4.90	0.39
	10%	6.62	2.68	4.10	3.83
Trametinib (MEK 1/2 inhibitor)	2%	0.02	0.01	0.17	0.12
	10%	6.29	0.105	0.04	>10.00
Selumetinib (MEK/ERK1/ERK2 inhibitor)	2%	> 10.00	6.72	> 10.00	>10.00
	10%	8.40	4.39	4.20	>10.00
Miransertib (AKT1/2/3 inhibitor)	2%	0.60	3.03	5.25	2.56
	10%	3.25	8.17	1.66	9.90
Lorlatinib (ALK/Ros1 inhibitor)	2%	>10.00	9.50	> 10.0	8.76
	10%	8.91	8.79	7.20	> 10.00
Docetaxel (depolymerisation of microtubules)	2%	1.97	0.692	0.11	1.04
	10%	0.002	1.797	1.44	1.25
Paclitaxel (microtubule polymer stabiliser)	2%	0.12	0.015	0.018	0.01
	10%	0.03	0.039	0.002	0.03

Each value is the mean of triplicate samples. IC_{50}: 50% inhibitory concentration; SRB: sulforhodamine B; TKIs: tyrosine kinase inhibitors; STAT: signal transducer and activator of transcription; Abl: Abelson murine leukemia viral oncogene homolog; AKT: serine/threonine protein kinase; ALK anaplastic lymphoma kinase; c-MET: hepatocyte growth factor receptor; CDK: cyclin dependent kinase; EGFR: epidermal growth factor receptor; EPGN: epithelial mitogen; FBS fetal bovine serum; FGFR: fibroblast growth factor receptor; HBCCLs: human brain cancer cell lines; HER: human epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; VEGFR: vascular endothelial growth factor.

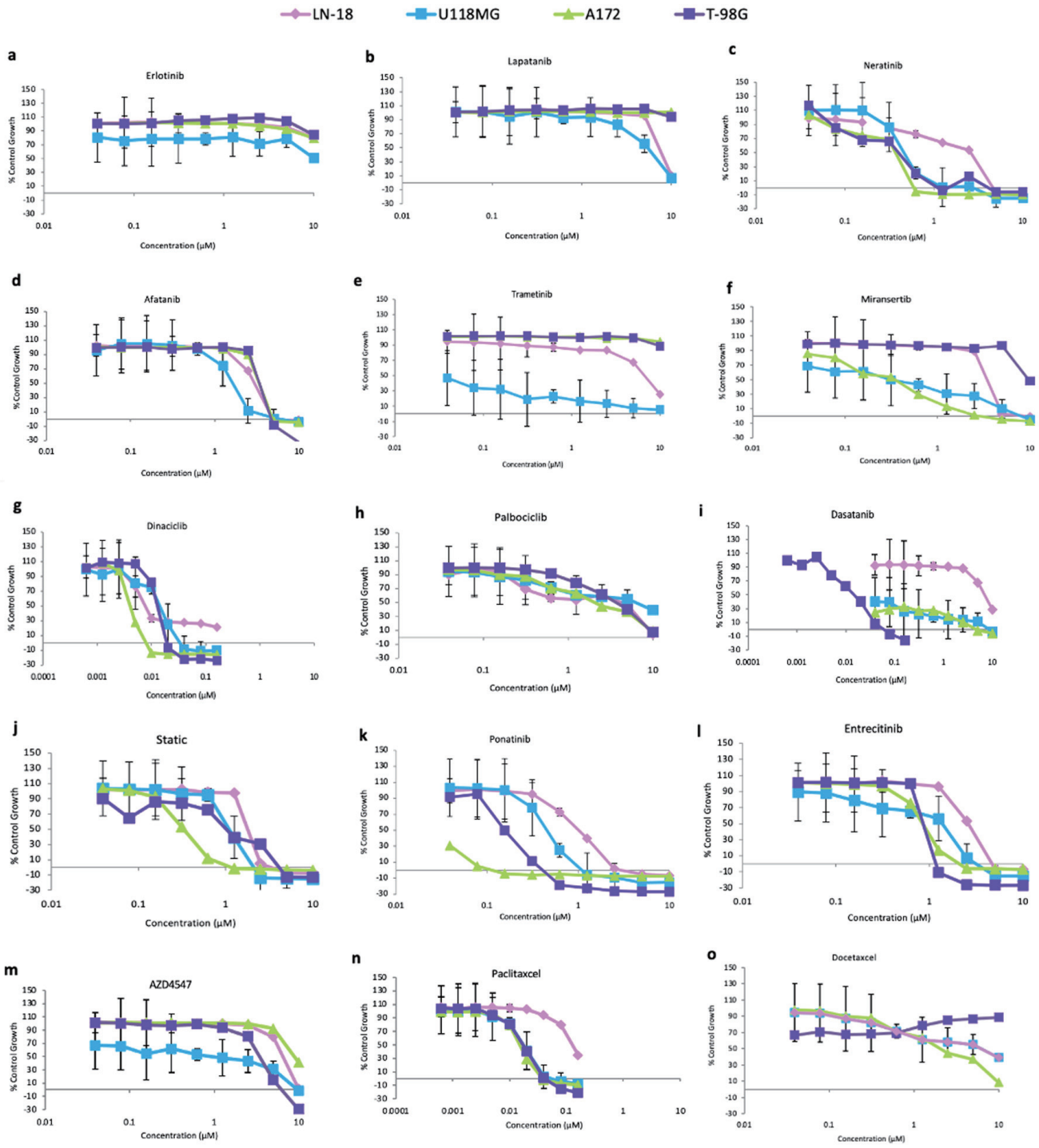

Figure 2. Effect of doubling dilutions of various agents targeting HER family members and other cell signaling molecules on the growth of brain cancer cells when cultured in medium containing 2% FBS. Tumor cells that were examined when grown in control wells (i.e., only medium) were confluent. Each point represents the mean ± SD of the triplicate sample. FBS fetal bovine serum; HER: human epidermal growth factor receptor; SD: standard deviation.

cuCyte, and results are presented in Figure 4. In comparison to the positive control (i.e., no treatment, 10% FBS medium only) treatment with drugs such as neratinib ($P = 0.016$), dinaciclib ($P = 0.013$), stattic ($P = 0.013$), dasatinib ($P = 0.017$), trametinib ($P = 0.045$) and paclitaxel ($P = 0.013$), was accompanied by reduction of the migration of A172 cells (Fig. 4). Values on the figure show whether there was statistical significance or no significance (NS).

Table 3. Effect on Cell Cycle Distribution on LN-18, U118MG, A172 and T-98G Cells Following Treatment With HER-Family Inhibitor Neratinib in Combination With Various Agents

Cell lines	Cell cycle phase (% of gated cells)				
	Treatment	Sub G1	G0/G1	S	G2/M
LN-18	Control	0.8 ± 0.02	73.7 ± 3.9	9.6 ± 0.9	15.07 ± 4.7
	Neratinib	11.9 ± 1.1	71.59 ± 2.8	6.2 ± 0.9	8.8 ± 2.3
	Dinaciclib	51.3 ± 7.5	33.5 ± 0.2	4.4 ± 1.1	9.7 ± 4.8
	Dasatinib	3.9 ± 1.1	82.8 ± 8.2	9.0 ± 0.8	10.5 ± 0.4
	Stattic	98.8 ± 0.3	0.81 ± 0.14	0.5 ± 0.01	0.15 ± 0.05
	Paclitaxel	11.63 ± 3.9	59.6 ± 10.7	15.9 ± 1.9	13.3 ± 1.5
U118MG	Control	6.9 ± 1.1	76.9 ± 6.9	12.2 ± 7.9	3.6 ± 0.7
	Neratinib	5.6 ± 4.4	84.4 ± 0.6	6.05 ± 3.6	3.5 ± 1.0
	Dinaciclib	42.7 ± 18.3	50.1 ± 13.3	5.4 ± 4.9	1.4 ± 0.3
	Dasatinib	8.2 ± 4.4	88.3 ± 3.1	2.1 ± 0.7	1.0 ± 0.7
	Stattic	37.5 ± 29.3	54.1 ± 19.8	8.1 ± 9.6	1.5 ± 1.7
	Paclitaxel	22.1 ± 9.8	70.3 ± 13.2	10.4 ± 7.5	2.9 ± 0.05
A172	Control	2.8 ± 0.9	90.4 ± 1.8	4.1 ± 0.3	2.5 ± 0.01
	Neratinib	45.1 ± 34.1	36.3 ± 13.3	18.5 ± 21.7	2.2 ± 2.2
	Dinaciclib	67.7 ± 7.3	25.8 ± 1.08	7.3 ± 7.7	0.2 ± 0.3
	Dasatinib	12.6 ± 5.6	72.3 ± 10.6	16.5 ± 20.5	1.3 ± 0.1
	Stattic	9.1 ± 9.6	82.2 ± 4.5	6.8 ± 5.5	2.7 ± 1.4
	Paclitaxel	55.6 ± 13.6	25.2 ± 6.4	12.4 ± 4.9	6.0- ± 3.2
T-98G	Control	1.9 ± 1.04	88.9 ± 2.3	6.7 ± 1.5	2.6 ± 0.4
	Neratinib	1.67 ± 0.7	83.1 ± 6.8	12.9 ± 8.2	3.2 ± 0.5
	Dinaciclib	28.8 ± 0.5	3.2 ± 0.07	5.07 ± 0.02	59.7 ± 0.6
	Dasatinib	14.1 ± 3.3	70.0 ± 2.0	13.4 ± 5.6	3.5 ± 0.9
	Stattic	48.3 ± 65.7	41.5 ± 52.7	8.9 ± 12.2	1.5 ± 1.9
	Paclitaxel	64.1 ± 18.8	25.8 ± 13.9	7.6 ± 2.9	1.9 ± 1.3

Each value is expressed as mean ± standard deviation (SD). HER: human epidermal growth factor receptor.

Treatment with a combination of neratinib with palbociclib or miransertib resulted in the synergistic growth inhibition of brain cancer cell lines

Next, the effect of neratinib in combination with other targeted agents on growth of HBCCLs were investigated. Treatment with the irreversible HER TKI neratinib in combination with the CDK4/6 inhibitor palbociclib resulted in the synergistic growth inhibition of all three HBCCLs (Table 4). In addition, treatment with a combination of neratinib and AZD4547, neratinib and trametinib, or neratinib with miransertib has resulted in synergistic or additive growth inhibition of these brain cancer cells lines. These effects were similar when the tumors were growing at both a higher rate (i.e., 10% serum) and slower rate (i.e., 2% serum) (Table 4). In contrast, treatment with a combination of neratinib with dinaciclib resulted in synergistic growth inhibition of T-98G cells, but the same combination was antagonistic in LN-18 and A172 cells (Table 4). Finally, treatment with neratinib in combination with the remaining targeted agents resulted in the synergistic growth inhibition of some of the HBCCLs, however the same combinations were antagonistic in other brain cancer cells.

The expression of CD44 may be of predictive value of the response to treatment with dasatinib and trametinib

The association between the expression level of various growth factor receptors and their response to treatment with various agents was assessed using SPSS software as shown in Table 5. There was no correlation between the expression level of EGFR and HER2 and the response to various targeted agents. The expression of HER3 and HER4 were negative in HBCCLs, and therefore the correlation study was not performed. However, there were some statistically significant associations between CD44 expression and the response to treatment with Abl/Src/c-Kit inhibitor dasatinib (R^2 = 0.904, P = 0.032) and MEK 1/2 inhibitor trametinib (R^2 = 0.904, P = 0.049), when human BCCLs were cultured in medium containing 10% and

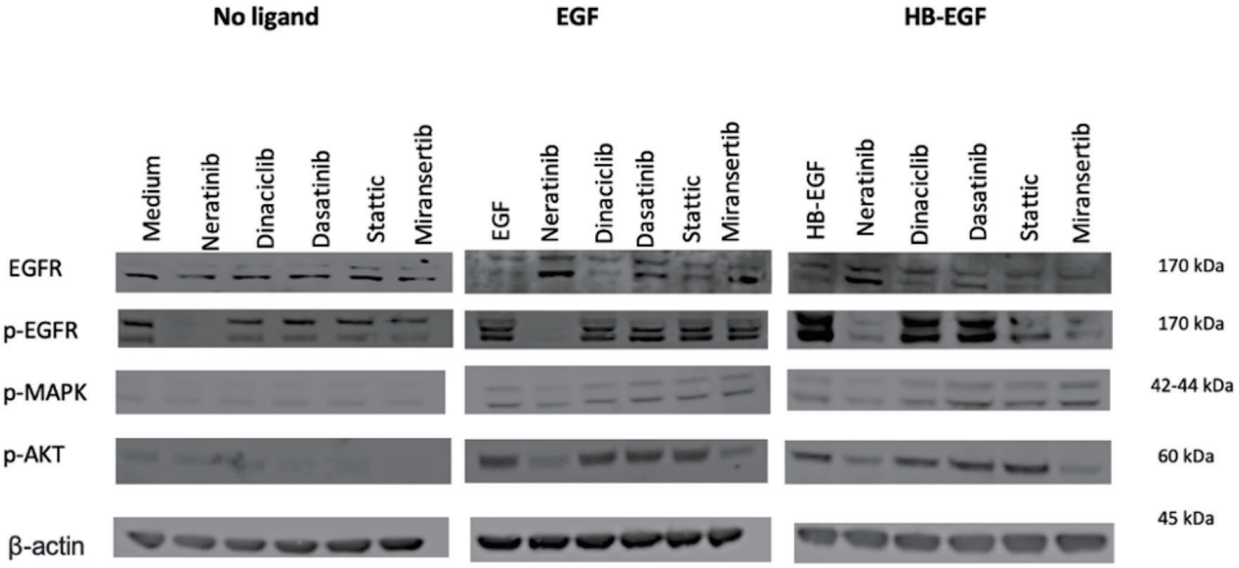

Figure 3. Effects of tyrosine kinase inhibitors (TKIs) on cell signaling on brain tumor cell line A172 in the presence or absence of ligands. The cells were cultured in 10% FBS DMEM medium to near confluency. Cells were washed once with 0.5% FBS DMEM medium and incubated with selected agents (400 nM) for 1 h and then stimulated with 30 nM ligands (EGF or HB-EGF) for 15 min. Cells were then lysed, separated using SDS-PAGE, transferred onto PVDF membranes, probed with the antibodies of interest, and visualized using LI-COR software. DMEM: Dulbecco's modified Eagles medium; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; EGF: epidermal growth factor; HB-EGF: heparin-binding EGF-like growth factor; EGFR: epidermal growth factor receptor; MAPK: mitogen-activated protein kinase; AKT: serine/threonine protein kinase; STAT3: signal transducer and activator of transcription 3; SRC: proto-oncogene tyrosine kinase SRC.

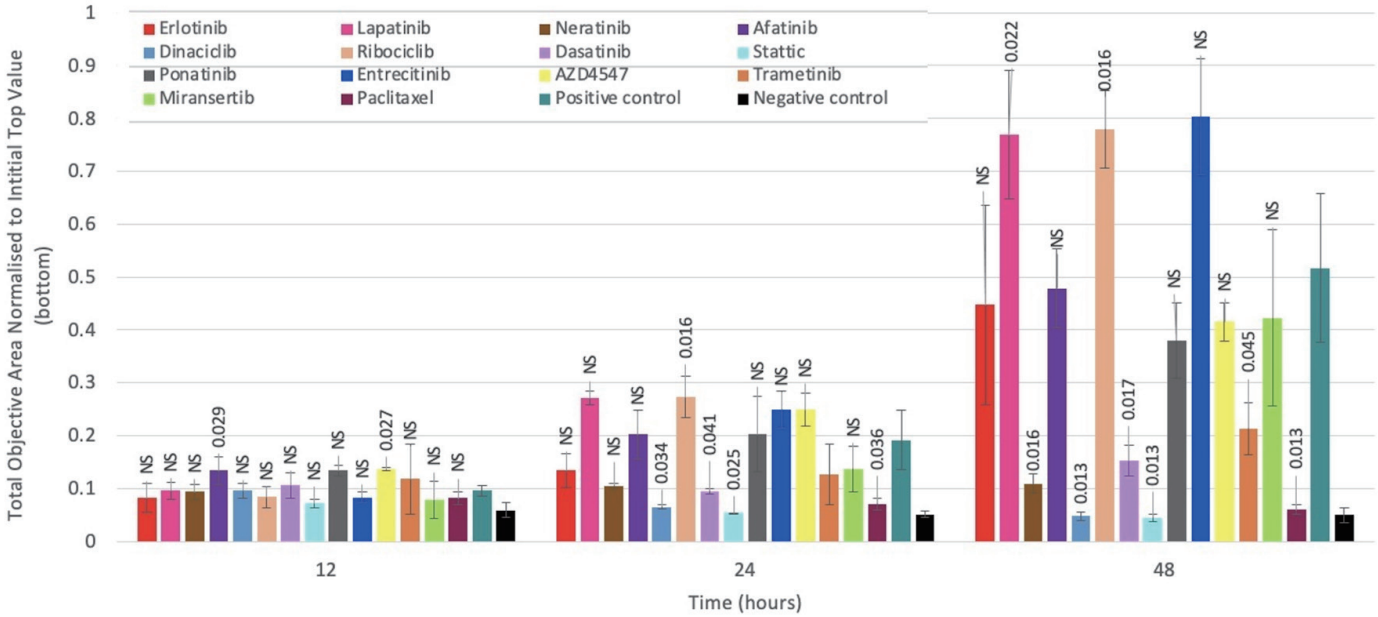

Figure 4. The effect of various agents on migration of brain cancer cell line A172. Migration is determined using the IncuCyte Clear View 96 well IncuCyte Chemotaxis system. Cells were seeded into the top layer of a 96-well cell migration assay plate in 0.5% FBS DMEM together with TKIs at IC_{50} concentrations, while 10% FBS DMEM (chemoattractant) was added to the bottom layer. Cells were incubated at 37 °C for 48 h, with images taken from chamber wells and were analyzed every 3 h for 48 h using the IncuCyte chemotaxis software. Most TKIs inhibited migration of A172 cell line, to varying degrees. Each point is a representative of the mean ± SD of triplicate samples. NS: not significant; IC_{50}: 50% inhibitory concentration; TKIs: tyrosine kinase inhibitors; SD: standard deviation; FBS fetal bovine serum; DMEM: Dulbecco's modified Eagles medium.

Table 4. The Effect of Treatment With the Pan-HER Family Inhibitor Neratinib When Used In Combination With Other Drugs on Cell Lines LN-18, A172 and T-98G

Drug combination		Combination index mean (range)					
		LN-18		A172		T-98G	
		10%	2%	10%	2%	10%	2%
Neratinib	Palbociclib	0.43	0.18	0.68	0.45	0.34	0.88
	Dinaciclib	3.78	1.14	1.12	1.06	0.84	0.61
	Capmatinib	0.28	0.35	1.47	1.15	0.60	0.78
	Dasatinib	0.75	1.78	1.82	0.71	0.75	0.88
	Stattic	0.83	0.71	0.72	1.2	9.17	0.78
	Ponatinib	0.63	0.97	3.1	0.97	0.99	0.85
	AZD4547	1.01	0.25	1.1	0.08	0.58	0.18
	Trametinib	0.89	0.77	1.02	1.15	0.91	0.26
	Miransertib	0.24	0.52	0.59	0.70	0.91	0.21
	Paclitaxel	0.34	1.44	1.27	0.87	1.37	0.85

Combination Index < 0.9 = synergistic effect, 0.9 - 1.1 = additive effect, > 1.1 = antagonistic. HER: human epidermal growth factor receptor.

2% serum respectively (Table 5).

Discussion

Despite the major advances in early diagnosis and treatment of various types of human cancers, brain cancer remains one of the most aggressive and lethal forms of cancer [1]. Currently, there are no curative treatment options for GB (glioblastoma multiforme (GBM)), and the survival rate of patients diagnosed with GBM remains one of the lowest for patients with various types of cancers [19]. In the past few decades, aberrant

Table 5. Linear Regression Analysis of the Expression of Various Receptors Against the Sensitivity of Human Brain Cancer Cell Lines to Treatment With Various TKIs, CDK Inhibitors, STAT3 Inhibitor and Cytotoxic Agents

Drugs/cell surface markers	2%			10%		
	EGFR	HER2	CD44	EGFR	HER2	CD44
	R^2 (P value)	R^2 (P value)	R^2 (P value)	R^2 (P value)	R^2 (P value)	R^2 (P value)
Erlotinib	0.784 (0.114)	0.717 (0.153)	0.221 (0.530)	N/A	N/A	N/A
Lapatinib	0.901 (0.051)	0.634 (0.204)	0.070 (0.735)	0.019 (0.863)	0.528 (0.273)	0.125 (0.647)
Neratinib	0.164 (0.595)	0.069 (0.737)	0.372 (0.390)	0.531 (0.271)	0.023 (0.850)	0.004 (0.941)
Afatinib	0.042 (0.796)	0.011 (0.897)	0.775 (0.120)	0.461 (0.321)	0.047 (0.783)	0.004 (0.939)
Palbociclib	0.107 (0.673)	0.031 (0.824)	0.694 (0.167)	0.065 (0.745)	0.796 (0.108)	0.004 (0.939)
Dinaciclib	0.023 (0.849)	0.811 (0.099)	0.064 (0.746)	0.543 (0.263)	0.003 (0.947)	0.102 (0.681)
Ribociclib	0.049 (0.778)	0.837 (0.085)	0.007 (0.916)	0.476 (0.310)	0.036 (0.810)	0.021 (0.855)
Dasatinib	0.226 (0.525)	0.460 (0.322)	0.814 (0.098)	0.152 (0.610)	0.275 (0.475)	0.904 (0.032)
Stattic	0.297 (0.455)	0.012 (0.891)	0.405 (0.364)	0.560 (0.251)	0.002 (0.961)	0.251 (0.499)
Ponatinib	0.002 (0.958)	0.328 (0.428)	0.656 (0.190)	0.000 (0.985)	0.479 (0.308)	0.475 (0.311)
Entrectinib	0.272 (0.479)	0.066 (0.743)	0.507 (0.288)	0.128 (0.643)	0.126 (0.644)	0.288 (0.464)
AZD4575	0.023 (0.850)	0.481 (0.307)	0.212 (0.539)	0.557 (0.254)	0.692 (0.168)	0.024 (0.846)
Trametinib	0.000 (0.992)	0.145 (0.619)	0.904 (0.049)	0.813 (0.098)	0.039 (0.803)	0.090 (0.700)
Selumetinib	N/A	N/A	N/A	0.856 (0.075)	0.051 (0.773)	0.052 (0.771)
Miransertib	0.455 (0.325)	0.008 (0.909)	0.308 (0.445)	0.001 (0.963)	0.610 (0.219)	0.000 (0.994)
Lorlatinib	0.047 (0.784)	0.303 (0.450)	0.139 (0.627)	0.310 (0.443)	0.136 (0.631)	0.005 (0.927)
Docetaxel	0.222 (0.529)	0.194 (0.560)	0.086 (0.707)	0.009 (0.953)	0.634 (0.204)	0.064 (0.747)
Paclitaxel	0.872 (0.066)	0.218 (0.533)	0.024 (0.846)	0.352 (0.407)	0.308 (0.445)	0.291 (0.461)

N/A: not available; TKIs: tyrosine kinase inhibitors; STAT: signal transducer and activator of transcription; CDK: cyclin dependent kinase; EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor; CD44: cluster differentiation 44.

expression and activation of HER family members have been reported in a wide range of cancers, and of these EGFR and HER2 are important targets for therapy with various types of monoclonal antibody-based products and small molecule tyrosine inhibitors [20, 21]. To date, TMZ is the only drug that has gained FDA approval for treatment for patients with brain cancer, and no HER inhibitors have yet been approved for the treatment of patients with brain cancers. The heterogenous nature of brain cancer (i.e., intra- and inter-tumor heterogeneity) and its microenvironment, and activation with other redundant cell signaling pathways could be some of the factors responsible for the primary or acquired resistance to treatment with the EGFR or other types of HER inhibitors in brain cancer [14, 22, 23].

Increased proliferation, dysregulated cell-cycle progression and increased migration and invasion are important hallmarks of human cancers [9]. Therefore, in this study and to our knowledge for the first time, we investigated the growth response of a panel of human GB cell lines to treatment with several agents targeting one or more members of the HER family, other growth factors receptor systems (e.g., PDGFRα and FGFR1/2/3, c-MET), different cell-cycle proteins (e.g., CDK4/6 inhibitor) and downstream cell signaling molecules (e.g., MEK1/2 or AKT1/2/3). Some of the agents investigated in this study, such as the irreversible pan-HER family blockers neratinib and afatinib or the CDK4/6 inhibitor palbociclib, have already been approved for the treatment of patients with other types of cancer, such as lung or breast cancers [24, 25]. Of the HER family inhibitors, treatment with neratinib and afatinib, which are irreversible pan-HER inhibitors, were more effective than treatment with reversible EGFR-specific erlotinib and reversible dual EGFR/HER-2 TKI lapatinib, in inhibiting growth for all four brain cancer cell lines. Moreover, treatment with neratinib also inhibited the ligand-induced phosphorylation of EGFR in A172 cells, which unlike other pHER2- and pHER3-positive human breast (SKBR3) and stomach (N-87) cancer cell lines, were pHER2- and pHER3-negative [26]. Indeed, neratinib has already been approved for the treatment of patients with HER2-positive breast cancer, who received two or more prior anti-HER2-based therapy [27]. Neratinib was found to be effective in the treatment of HER2-positive breast cancer with brain metastases although no significant benefit was seen in quality of life due to the toxicity [28]. More recently, the OS after anti-HER2 trastuzumab therapy (ExtesNET) in HER2-positive breast cancer in the extended adjuvant setting was found to be comparable for neratinib and placebo, after a median follow-up of 8.1 years. These studies highlight the importance of investigating the therapeutic benefit of neratinib in combination with agents against other cells signaling pathways and molecules in human cancers [29]. In another study, using an unbiased computational prioritization coupled with high-throughput screening, Houweling et al examined treatment of 25 patient-derived GBM cultures, with 15 out of 43 drug combinations resulting in synergistic interaction. In particular, they found that treatment with a combination of dual EGFR/HER2 inhibitor lapatinib and thapsigargin (Ca^{2+} ATPase) and lapatinib with obatoclax mesylate (BCL2 and MCL1) resulted in highest synergistic effect, with the apoptosis pathway being suggested to be one of the mechanisms involved in long-term effect in GBM cells [30].

A common feature of almost all human cancers is the dysregulation and over activation of CDKs, and to date three different CDK4/6 inhibitors have been approved for the treatment of metastatic hormone receptor-positive breast cancer [31]. Therefore, in this study we examined the effect of different CDK inhibitors on the growth of brain cancer cell lines when used alone or in combination with the irreversible pan-HER inhibitor neratinib. Of all agents examined, the CDK1/2/5/9 inhibitor dinaciclib was the most potent agent and inhibited the proliferation of all four HBCCLs more effectively than the CDK4/6 inhibitors palbociclib and ribociclib (Table 2). In another study, Buzzetti et al have also found that dinaciclib was more effective than palbociclib in inhibiting the growth of medulloblastoma cells [32]. Interestingly, while treatment with neratinib in combination with dinaciclib resulted in slight to moderate antagonism in two of the HBCCLs, treatment with a combination of neratinib and palbociclib resulted in synergistic growth inhibition of all three HBCCLs (Table 4). As both neratinib and palbociclib have already been approved for the treatment of patient with breast cancer, and treatment with a combination of these two drugs resulted in synergistic growth inhibition of all three HBCCLs, treatment with neratinib in combination with palbociclib may also be of therapeutic value in brain cancer by drug repurposing. Currently, clinical trials with both agents as monotherapy are underway in patients with GB (NCT02977780 and NCT05432518). Therefore, further investigation is warranted to determine the therapeutic potential of such drugs when used in combination [33, 34].

The downstream signaling protein Src is a known key signaling pathway inducer of several membrane bound activated receptors, including EGFR. SRC/c-kit/Abl inhibitor dasatinib inhibited the growth of all four HBCCLs with IC_{50} of ≤ 3 μM. Interestingly, treatment with dasatinib was more effective in inhibiting the growth of HBCCLs when they were growing at slower rate (i.e., in medium containing lower serum concentration). In one study, treatment with dasatinib monotherapy in a phase II clinical trial in target-selected patients with recurrent GB was found to be ineffective, supporting the importance of treatment with a combination of agents [35]. Indeed, treatment with dasatinib in combination with TMZ resulted in clinical benefits in children with central nervous system tumors [36]. In this study, we found that treatment with a combination of neratinib and dasatinib resulted in synergistic growth inhibition in T-98G investigated under different serum concentration. However, the same combination was found to be antagonistic in LN-18 and A172 cells, which were cultured in the medium containing 2% and 10% serum, respectively, highlighting the complex and heterogeneous nature of brain cancer (Table 4).

CD44 has been shown to be an important biomarker in many types of cancers and is a putative brain cancer stem cell marker. In one study, Wang et al found CD44 expression may be associated with invasion and migration of glioma [37]. In this study, we found the expression of CD44 to be high in three of the four human brain cancer cell lines (Table 1). Notably, we found a statistically significant association between the expression CD44 and response of HBCCLs to the treatment with dasatinib when such cancer cells were grown at higher

rate (Table 5). Therefore, our results suggest that expression of CD44 may be of potential predictive value for the response to treatment to dasatinib in brain cancer.

Of the other targeted agents employed in our study, the AKT1/2/3 inhibitor miransertib inhibited the growth of all HBCCLs as a single agent. In particular, treatment with a combination of neratinib with miransertib was accompanied by the synergistic growth inhibition of all HBCCLs (Table 4). Treatment with miransertib also reduced the EGF and HB-EGF phosphorylation of AKT in A172 cells (Fig. 3). Moreover, in another study, we found that treatment with a combination of the irreversible pan-HER blocker afatinib and miransertib resulted in synergistic growth inhibition of a panel of human gastric cancer cell lines [26]. While AKT pathway is one of the critical signaling outputs of EGFR and HER pathways, to our knowledge there has been no study of the therapeutic potential of the irreversible pan-HER family inhibitor such as neratinib when used in combination with miransertib in brain cancer [38]. Taken together, our results support that treatment with a combination of the irreversible pan-HER family blocker neratinib in combination with the AKT inhibitor miransertib may be of therapeutic value in brain cancer.

The coexpression of other growth factor receptors and over-activation of downstream cell signaling molecules have been associated with resistance of tumor cells to treatment with the HER inhibitors and cytotoxic drugs [12, 39-42]. The expression levels of ALK and c-MET were found to be very low in brain cancer cells, which is in concordance with the poor response of all four HBCCLs to the treatment with the c-MET inhibitor (capmatinib) and ALK inhibitor (lorlatinib) (Table 2). However, neratinib in combination with c-MET TKI capmatinib resulted in synergistic growth inhibition of LN-18 and T-98 cells, but the same combination was antagonistic in A172 cells, highlighting the complex biology and heterogeneous nature of brain cancer. While there has been no study of treatment with a combination of neratinib and capmatinib in patients with brain cancer, there is currently an ongoing clinical trial (NCT05243641) examining of this combinational treatment in patients with breast cancer, and the results should reveal the therapeutic potential of such combinations in patients with other solid tumors. Finally, in addition to increased proliferation, increased motility is another hallmark of human cancers. Therefore, in this study, we examined the effects of these targeted agents on the migration of the brain cancer cell line A172 cells, which was found to be the only migratory cell line. We found that treatments with the targeted agents such as stattic, neratinib, trametinib were more effective than other targeted agents by inhibiting the migration of A172 cells (Fig. 4).

In conclusion, of targeting agents employed in this study, we found that the irreversible pan-HER TKI neratinib, CDK1/2/5/9 inhibitor dinaciclib and the Src targeting TKI dasatinib were the most effective at inhibiting the growth of brain cancer cells, blocking cell signaling through HER family members and reducing the migration of brain cancer cells. Interestingly, treatment with the irreversible pan-HER family blocker neratinib in combination with the CDK4/6 inhibitor palbociclib or the AKT1/2/3 inhibitor miransertib resulted in synergistic growth inhibition of all HBBCLs. We also found that the CD44 expression may be a predictive factor of the response to treatment with stattic and trametinib. Taken together, our results suggest that treatment with the irreversible pan-HER inhibitor neratinib when used in combination with the CDK4/6 inhibitor palbociclib or the AKT1/2/3 miransertib may be of therapeutic value in brain cancer by repurposing such drugs.

Acknowledgments

We would like to acknowledge and thank Kingston University London for providing a PhD bursary.

Financial Disclosure

This study was supported by the Kingston University as part of a self-funded PhD project at the Kingston University London, UK.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent

Not applicable.

Author Contributions

Helmout Modjtahedi is Ermira Mulliqi's director of studies, and provided study concept, design, data analysis and critical revision of the manuscript. Ermira Mulliqi performed all the experiments, data analysis and drafting of manuscript. Dr. Khelwatty, Dr. Morgan, and Professor Ashkan are the cosupervisors on the project and did critical revision of the manuscript. Dr. Khelwatty also helped with the training of various techniques and data analysis.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

Abl: Abelson murine leukemia viral oncogene homolog; AKT: serine/threonine protein kinase; ALK, anaplastic lymphoma kinase; AREG, amphiregulin; BTC: betacellulin; c-MET: hepatocyte growth factor receptor; CD: cluster differentiation; CDK: cyclin dependent kinase; DMSO: dimethyl sulfoxide; EGFR: epidermal growth factor receptor; EPGN: epithelial

mitogen; FBS: fetal bovine serum; FGFR: fibroblast growth factor receptor; FL PVDF: polyvinylidene fluoride; HBCCLs: human brain cancer cell lines; HER: human epidermal growth factor receptor; MFI: mean fluorescence intensity; PDGFR: platelet-derived growth factor receptor; ROS: ROS proto-oncogene 1 receptor tyrosine kinase; TGF: transforming growth factor; TRK: tropomyosin receptor kinase; VEGFR: vascular endothelial growth factor

References

1. Miller KD, Ostrom QT, Kruchko C, Patil N, Tihan T, Cioffi G, Fuchs HE, et al. Brain and other central nervous system tumor statistics, 2021. CA Cancer J Clin. 2021;71(5):381-406. doi pubmed
2. Zele T, Velnar T, Koritnik B, Bosnjak R, Markovic-Bozic J. Awake craniotomy for operative treatment of brain gliomas - experience from University Medical Centre Ljubljana. Radiol Oncol. 2023;57(2):191-200. doi pubmed pmc
3. Wu W, Klockow JL, Zhang M, Lafortune F, Chang E, Jin L, Wu Y, et al. Glioblastoma multiforme (GBM): An overview of current therapies and mechanisms of resistance. Pharmacol Res. 2021;171:105780. doi pubmed pmc
4. Wagle N, Kesari S. Breaking down the blood-brain barrier. Neuro Oncol. 2021;23(1):6. doi pubmed pmc
5. Aldape K, Brindle KM, Chesler L, Chopra R, Gajjar A, Gilbert MR, Gottardo N, et al. Challenges to curing primary brain tumours. Nat Rev Clin Oncol. 2019;16(8):509-520. doi pubmed pmc
6. Sergina NV, Moasser MM. The HER family and cancer: emerging molecular mechanisms and therapeutic targets. Trends Mol Med. 2007;13(12):527-534. doi pubmed pmc
7. Ioannou N, Seddon AM, Dalgleish A, Mackintosh D, Modjtahedi H. Expression pattern and targeting of HER family members and IGF-IR in pancreatic cancer. Front Biosci (Landmark Ed). 2012;17(7):2698-2724. doi pubmed
8. Singh B, Carpenter G, Coffey RJ. EGF receptor ligands: recent advances. F1000Res. 2016;5:2270. doi pubmed pmc
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-674. doi pubmed
10. Zubair T, Bandyopadhyay D. Small molecule EGFR inhibitors as anti-cancer agents: discovery, mechanisms of action, and opportunities. Int J Mol Sci. 2023;24(3):2651. doi pubmed pmc
11. Chong CR, Janne PA. The quest to overcome resistance to EGFR-targeted therapies in cancer. Nat Med. 2013;19(11):1389-1400. doi pubmed pmc
12. Yamaoka T, Ohba M, Ohmori T. Molecular-targeted therapies for epidermal growth factor receptor and its resistance mechanisms. Int J Mol Sci. 2017;18(11)2420. doi pubmed pmc
13. Talukdar S, Emdad L, Das SK, Fisher PB. EGFR: An essential receptor tyrosine kinase-regulator of cancer stem cells. Adv Cancer Res. 2020;147:161-188. doi pubmed
14. Torrisi F, Alberghina C, D'Aprile S, Pavone AM, Longhitano L, Giallongo S, Tibullo D, et al. The Hallmarks of Glioblastoma: Heterogeneity, Intercellular Crosstalk and Molecular Signature of Invasiveness and Progression. Biomedicines. 2022;10(4):806. doi pubmed pmc
15. Roskoski R, Jr. Small molecule inhibitors targeting the EGFR/ErbB family of protein-tyrosine kinases in human cancers. Pharmacol Res. 2019;139:395-411. doi pubmed
16. Pineda E, Domenech M, Hernandez A, Comas S, Balana C. Recurrent Glioblastoma: Ongoing Clinical Challenges and Future Prospects. Onco Targets Ther. 2023;16:71-86. doi pubmed pmc
17. Khan T, Seddon AM, Dalgleish AG, Khelwatty S, Ioannou N, Mudan S, Modjtahedi H. Synergistic activity of agents targeting growth factor receptors, CDKs and downstream signaling molecules in a panel of pancreatic cancer cell lines and the identification of antagonistic combinations: Implications for future clinical trials in pancreatic cancer. Oncol Rep. 2020;44(6):2581-2594. doi pubmed pmc
18. Puvanenthiran S, Essapen S, Seddon AM, Modjtahedi H. Impact of the putative cancer stem cell markers and growth factor receptor expression on the sensitivity of ovarian cancer cells to treatment with various forms of small molecule tyrosine kinase inhibitors and cytotoxic drugs. Int J Oncol. 2016;49(5):1825-1838. doi pubmed pmc
19. Ostrom QT, Gittleman H, Xu J, Kromer C, Wolinsky Y, Kruchko C, Barnholtz-Sloan JS. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2009-2013. Neuro Oncol. 2016;18(suppl_5):v1-v75. doi pubmed pmc
20. Modjtahedi H, Ali S, Essapen S. Therapeutic application of monoclonal antibodies in cancer: advances and challenges. Br Med Bull. 2012;104:41-59. doi pubmed
21. Esparis-Ogando A, Montero JC, Arribas J, Ocana A, Pandiella A. Targeting the EGF/HER Ligand-Receptor System in Cancer. Curr Pharm Des. 2016;22(39):5887-5898. doi pubmed
22. Ou A, Yung WKA, Majd N. Molecular Mechanisms of Treatment Resistance in Glioblastoma. Int J Mol Sci. 2020;22(1):351. doi pubmed pmc
23. Dapash M, Hou D, Castro B, Lee-Chang C, Lesniak MS. The interplay between glioblastoma and its microenvironment. Cells. 2021;10(9):2257. doi pubmed pmc
24. Serra F, Lapidari P, Quaquarini E, Tagliaferri B, Sottotetti F, Palumbo R. Palbociclib in metastatic breast cancer: current evidence and real-life data. Drugs Context. 2019;8:212579. doi pubmed pmc
25. Ahn ER, Mangat PK, Garrett-Mayer E, Halabi S, Dib EG, Haggstrom DE, Alguire KB, et al. Palbociclib in patients with non-small-cell lung cancer with CDKN2A alterations: results from the targeted agent and profiling utilization registry Study. JCO Precis Oncol. 2020;4:757-766. doi pubmed
26. Al-Janaby T, Nahi N, Seddon A, Bagwan I, Khelwatty S, Modjtahedi H. The Combination of Afatinib With Dasatinib or Miransertib Results in Synergistic Growth Inhibition of Stomach Cancer Cells. World J Oncol. 2024;15(2):192-208. doi pubmed pmc
27. Nagpal A, Redvers RP, Ling X, Ayton S, Fuentes M, Tavancheh E, Diala I, et al. Neoadjuvant neratinib promotes

ferroptosis and inhibits brain metastasis in a novel syngeneic model of spontaneous HER2(+ve) breast cancer metastasis. Breast Cancer Res. 2019;21(1):94. doi pubmed pmc
28. Dekker TJA. Neratinib in HER2-positive breast cancer with brain metastases. J Clin Oncol. 2021;39(3):251-252. doi pubmed
29. Holmes FA, Moy B, Delaloge S, Chia SKL, Ejlertsen B, Mansi J, Iwata H, et al. Overall survival with neratinib after trastuzumab-based adjuvant therapy in HER2-positive breast cancer (ExteNET): A randomised, double-blind, placebo-controlled, phase 3 trial. Eur J Cancer. 2023;184:48-59. doi pubmed
30. Houweling M, Giczewska A, Abdul K, Nieuwenhuis N, Kucukosmanoglu A, Pastuszak K, Buijsman RC, et al. Screening of predicted synergistic multi-target therapies in glioblastoma identifies new treatment strategies. Neurooncol Adv. 2023;5(1):vdad073. doi pubmed pmc
31. Mughal MJ, Bhadresha K, Kwok HF. CDK inhibitors from past to present: A new wave of cancer therapy. Semin Cancer Biol. 2023;88:106-122. doi pubmed
32. Buzzetti M, Morlando S, Solomos D, Mehmood A, Cox AWI, Chiesa M, D'Alessandra Y, et al. Pre-therapeutic efficacy of the CDK inhibitor dinaciclib in medulloblastoma cells. Sci Rep. 2021;11(1):5374. doi pubmed pmc
33. Zhang Z, Zhou L, Xie N, Nice EC, Zhang T, Cui Y, Huang C. Overcoming cancer therapeutic bottleneck by drug repurposing. Signal Transduct Target Ther. 2020;5(1):113. doi pubmed pmc
34. Zhao M, Scott S, Evans KW, Yuca E, Saridogan T, Zheng X, Wang H, et al. Combining neratinib with CDK4/6, mTOR, and MEK inhibitors in models of her2-positive cancer. Clin Cancer Res. 2021;27(6):1681-1694. doi pubmed pmc
35. Lassman AB, Pugh SL, Gilbert MR, Aldape KD, Geinoz S, Beumer JH, Christner SM, et al. Phase 2 trial of dasatinib in target-selected patients with recurrent glioblastoma (RTOG 0627). Neuro Oncol. 2015;17(7):992-998. doi pubmed pmc
36. Robison NJ, Yeo KK, Berliner AP, Malvar J, Sheard MA, Margol AS, Seeger RC, et al. Phase I trial of dasatinib, lenalidomide, and temozolomide in children with relapsed or refractory central nervous system tumors. J Neurooncol. 2018;138(1):199-207. doi pubmed pmc
37. Wang HH, Liao CC, Chow NH, Huang LL, Chuang JI, Wei KC, Shin JW. Whether CD44 is an applicable marker for glioma stem cells. Am J Transl Res. 2017;9(11):4785-4806. pubmed pmc
38. Hua H, Zhang H, Chen J, Wang J, Liu J, Jiang Y. Targeting Akt in cancer for precision therapy. J Hematol Oncol. 2021;14(1):128. doi pubmed pmc
39. Guo G, Narayan RN, Horton L, Patel TR, Habib AA. The role of EGFR-met interactions in the pathogenesis of glioblastoma and resistance to treatment. Curr Cancer Drug Targets. 2017;17(3):297-302. doi pubmed
40. Liu Q, Yu S, Zhao W, Qin S, Chu Q, Wu K. EGFR-TKIs resistance via EGFR-independent signaling pathways. Mol Cancer. 2018;17(1):53. doi pubmed pmc
41. Xu B, Huo Z, Huang H, Ji W, Bian Z, Jiao J, Sun J, et al. The expression and prognostic value of the epidermal growth factor receptor family in glioma. BMC Cancer. 2021;21(1):451. doi pubmed pmc
42. Xu H., et al. CD44 as a tumor biomarker and therapeutic target. Springer Science and Business Media LLC. 2020.