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The ALK inhibitors, alectinib and ceritinib, induce ALK-independent and STAT3-dependent glioblastoma cell death

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Abstract

Glioblastoma (GBM) is the most common, but extremely malignant, brain tumor; thus, the development of novel therapeutic strategies for GBMs is imperative. Many tyrosine kinase inhibitors (TKIs) have been approved for various cancers, yet none has demonstrated clinical benefit against GBM. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) that is confirmed only during the embryonic development period in humans. In addition, various ALK gene alterations are known to act as powerful oncogenes and therapeutic targets in various tumors. The antitumor activity of various TKIs was tested against three human GBM cell lines (U87MG, LN229, and GSC23), which expressed substantially low ALK levels; second-generation ALK inhibitors, alectinib and ceritinib, effectively induced GBM cell death. In addition, treatment with either alectinib or ceritinib modulated the activation of various molecules downstream of RTK signaling and induced caspase-dependent/-independent cell death mainly by inhibiting signal transducer and activator of transcription 3 activation in human GBM cells. In addition, alectinib and ceritinib also showed antitumor activity against a U87MG cell line with acquired temozolomide resistance. Finally, oral administration of alectinib and ceritinib prolonged the survival of mice harboring intracerebral GBM xenografts compared with controls. These results suggested that treatment with the second-generation ALK inhibitors, alectinib and ceritinib, might serve as a potent therapeutic strategy against GBM.

KEYWORDS

alectinib, anaplastic lymphoma kinase, ceritinib, glioblastoma, STAT3 transcription factor

Abbreviations: ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; GBM, glioblastoma; InsR, insulin receptor; JAK2, janus kinase 2; NPM, nucleophosmin; NSCLC, non–small cell lung carcinoma; PARP, poly ADP-ribose polymerase; RET, rearranged during transfection; RTK, receptor tyrosine kinase; STAT3, signal transducer and activator of transcription 3; TKI, tyrosine kinase inhibitor; TMZ, temozolomide; z-VAD, zVAD-FMK.

1 | INTRODUCTION

Glioblastoma (GBM) is the most common, but extremely malignant, brain tumor.[1] With the current standard treatment, maximal safe tumor resection followed by chemoradiotherapy with temozolomide (TMZ) against the residual tumor, the recurrence of GBMs is inevitable in most cases; the median overall survival is less than two years,[2] and this poor prognosis has not been significantly improved. In the past decades, many tyrosine kinase inhibitors (TKIs) targeting specific receptor tyrosine kinases (RTKs) have been approved for various cancers,[3] and in recent years, some TKIs have been investigated for the development of a novel therapeutic strategy for GBM. However, no TKI has demonstrated clinical benefit so far.[4]

Anaplastic lymphoma kinase (ALK) is an RTK that was initially discovered as a fusion nucleophosmin (*NPM*)-*ALK* gene in anaplastic large cell lymphoma (ALCL).[5] Under normal conditions, human ALK expression is confirmed only during the embryonic development period, and its physiological function is unclear, as ALK knockout mice do not exhibit any morphological defect or infertility.[6,7] Recent studies have revealed a variety of *ALK* alterations, such as oncogenic fusion,[8,9] activating point mutation,[10] and wild-type gene amplification,[11] that act as powerful oncogenes in various tumors. Importantly, together with the characteristic that normal wild-type ALK is not expressed in adult humans, as mentioned above, these *ALK* mutations are also suggested as potent tumor-specific therapeutic targets, and ALK inhibitors have been approved and used for clinical treatment.[12] The second-generation ALK inhibitor alectinib, which was approved for the treatment of advanced ALK-positive non–small cell lung carcinoma (NSCLC) in 2015, is known to block ALK activity with high specificity.[13] In the study of *EML4-ALK*–positive NSCLC and *NPM-ALK*–positive ALCL, treatment with alectinib subsequently constrains the activation of signal transducer and activator of transcription 3 (STAT3) and protein kinase B (AKT) in these cells and suppresses cell growth.[13] In another study investigating the safety and activity of alectinib against brain metastasis of NSCLC, 52% of patients experience partial response according to response assessment criteria of the RECIST version 1.1.[14] Ceritinib is another second-generation ALK inhibitor that was approved for the treatment of ALK-positive NSCLC in 2014. As a feature of ceritinib, this agent is orally available and possesses high selectivity against ALK, 20-fold more than the first-generation ALK inhibitor, crizotinib.[15] In addition, a multicenter phase II trial also revealed a 45% objective intracranial response in brain metastasis cases upon RECIST version 1.1 assessment.[16]

In this study, the effects of various TKIs against GBM cell lines were investigated. Among them, it was found that second-generation ALK inhibitors, alectinib and ceritinib, effectively induced GBM cell death. Therefore, their antitumor activities were further studied against multiple human GBM cells in vitro and in vivo. Alectinib and ceritinib effectively induced cell death in established and patient-derived human GBM cell lines, even without ALK expression and activation. In addition, alterations of the multiple RTK downstream signaling pathways were observed by alectinib or ceritinib treatment against GBM cells. Furthermore, alectinib and ceritinib exhibited antitumor efficacy against TMZ-resistant GBM cells. Finally, alectinib and ceritinib prolonged survival in an intracranial xenograft mouse model using U87MG GBM cells. These results suggest the possibility of second-generation ALK inhibitors as novel GBM therapeutic reagents.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Reagents and antibodies used in this study are described in the Materials and Methods S1.

2.2 | Cell culture

Details of cell lines, culture media formulation, and culture conditions are described in the Materials and Methods S1.

2.3 | Cell death assay

Cellular nuclei stained with 100 mmol/L Hoechst 33 342 and 4.0 mg/mL propidium iodide (PI; Thermo Fisher Scientific) were counted using a fluorescence microscope (IX81-ZDC-DSU; Olympus), three independent times. At least 500 cells per sample were examined to calculate the ratio of PI-positive dead cells from the Hoechst-positive total number of cells.

2.4 | Cell proliferation and cytotoxicity assay

Cell proliferation and cytotoxicity assays were performed using the Cell Counting Kit 8 (CCK-8, Do Jindo Molecular Technologies). The cells were cultured in 96-well plates overnight and incubated with various concentrations of the compounds for 48 hours. The assays were performed three independent times, following the manufacturer's protocol.

2.5 | RNA isolation, cDNA synthesis, and quantitative real-time PCR

Details of RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis are described in the Materials and Methods S1.

2.6 | Immunoblot analysis

Immunoblot analysis details are described in the Materials and Methods S1.

2.7 | Immunohistochemistry

Details of immunohistochemistry are described in the Materials and Methods S1.

2.8 | Small interfering RNA (siRNA)-mediated gene knockdown

siRNA-mediated gene knockdown was performed by transfection of 20 nmol/L of Stealth Select RNAi (Thermo Fisher Scientific) using the Lipofectamine RNAiMAX Transfection Reagent (#13778075, Invitrogen). Details of siRNAs used in this study are described in the Materials and Methods S1.

2.9 | Animals and tumor models

Details of animals and tumor models are described in the Materials and Methods S1.

2.10 | Statistical analysis

The cytotoxicity of various TKIs, including alectinib and ceritinib, for the different cell lines obtained from the in vitro dye-exclusion method were compared using two-way analysis of variance (ANOVA). A comparison of cell death with or without the pan-caspase inhibitor, Z-VAD-FMK, obtained from the in vivo dye-exclusion method, was performed using ANOVA. Results of in vivo survival assays were compared using the log-rank test. A P-value < 0.05 was considered significantly different. All analyses were performed using GraphPad Prism 9 software (GraphPad Software).

3 | RESULTS

3.1 | The ALK inhibitors, alectinib and ceritinib, specifically induced human GBM cell death

First, to investigate the cytotoxic effects of TKIs in GBM cells, U87MG and LN229 human GBM cell lines were treated with the six single-targeted TKIs with different RTKs (erlotinib [epidermal growth factor receptor (EGFR) inhibitor], AG1478 [EGFR/phosphatidylinositol 4-kinase alpha inhibitor], linsitinib [insulin-like growth factor 1 receptor (IGF1R)/insulin receptor (InsR) inhibitor], imatinib [platelet-derived growth factor receptor (PDGFR)/breakpoint cluster region-ABL protooncogene 1 (BCR-ABL)/c-Kit inhibitor], and two ALK inhibitors [alectinib and ceritinib]). The genetic characteristics of these GBM cell lines used were previously investigated,[17] and these cells did not harbor any isocitrate dehydrogenase or RTK mutations. After 48 hours of treatment, cell death was quantified as described in the Materials and Methods. Among these TKIs, only alectinib and ceritinib effectively induced cell death in both U87MG and LN229 cells (Figure 1). These results suggested specific antitumor effects of the ALK inhibitors in GBM cells among various TKIs.

3.2 | The second-generation ALK inhibitors, alectinib and ceritinib, inhibited the proliferation of human GBM cell lines

Next, the effects of the second-generation ALK inhibitors, alectinib and ceritinib, against three human GBM cell lines (including an additional patient-derived human GBM cell line [GSC23]) were investigated to further generalize the antitumor effects of ALK inhibitors against human GBM cells. The effects of both alectinib and ceritinib on cellular proliferation of these three cell lines were assessed using the CCK-8 assay. Both alectinib and ceritinib inhibited the proliferation of all three GBM cell lines in a dose-dependent manner after 48 hours of treatment (Figure 2). In addition, the IC_{50} values of ceritinib were consistently lower than those of alectinib, suggesting ceritinib's higher antitumor efficacy against GBM cells. Collectively, these data demonstrated the antitumor effects of ALK inhibitors against GBM cells.

3.3 | ALK expression in human GBM cells was substantially low

Previous studies have reported that ALK expression levels in GBM cell lines are generally low.[18-20] Therefore, the question was asked how ALK was expressed and activated in the GBM cells used in this study. To determine this, ALK mRNA and protein levels in the three GBM cell lines used in the above experiments were investigated. Quantitative PCR demonstrated moderate ALK mRNA expression in U87MG cells compared with Nagai cells, a neuroblastoma cell line harboring amplified wild-type ALK with addiction, used as a positive control in this study. However, in the other cell lines, ALK mRNA expression was substantially lower than in U87MG and Nagai cells (Figure 3A). In addition, immunoblot analysis demonstrated that ALK protein expression was detectable in U87MG cells at a weak level, but was almost undetectable in the other three cell lines compared with Nagai cells (Figure 3B). Further, tyrosine phosphorylation of the ALK kinase domain, recognized as an activation barometer of ALK, was not confirmed in any of the three GBM cell lines, whereas this event was observed in Nagai cells and blocked by alectinib or ceritinib treatment (Figure 3B). These results suggested that alectinib and ceritinib might induce cell death in the three GBM cell lines via ALK expression/activity-independent machinery.

3.4 | Alectinib and ceritinib induced caspase-dependent/-independent cell death in human GBM cells

The above data prompted the exploration of how alectinib and ceritinib induced GBM cell death. Hence, the type of GBM cell death

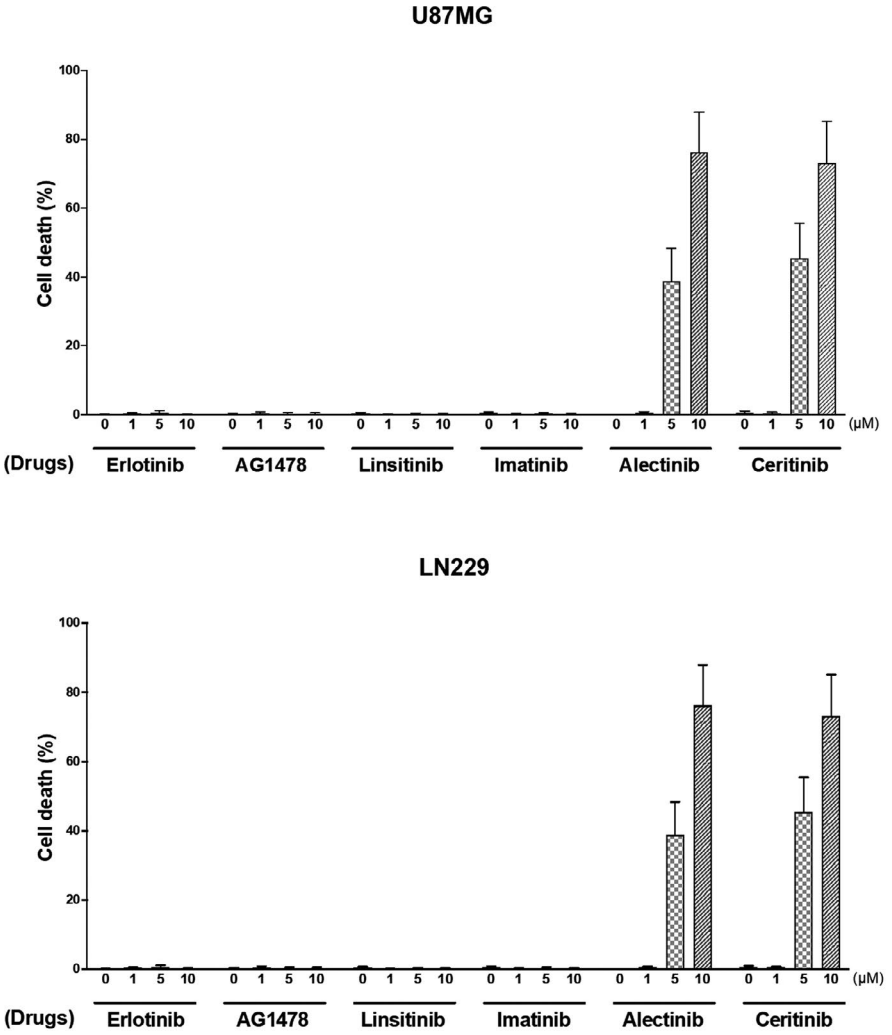


FIGURE 1 The second-generation anaplastic lymphoma kinase (ALK) inhibitors, alectinib and ceritinib, specifically induce glioblastoma (GBM) cell death. Human GBM cell lines, U87MG and LN229, were treated with DMSO (vehicle) or the indicated doses of erlotinib (epidermal growth factor receptor [EGFR] inhibitor), AG1478 (EGFR/phosphatidylinositol 4-kinase alpha [PI4KA] inhibitor), linsitinib (insulin-like growth factor 1 receptor [IGF-1R]/insulin receptor [InsR] inhibitor), imatinib (breakpoint cluster region-ABL protooncogene 1 [BCR-ABL]/c-Kit/platelet-derived growth factor receptor [PDGFR] inhibitor), alectinib (ALK/rearranged during transfection [RET] inhibitor), or ceritinib (ALK inhibitor). After 48 h, the mortality rates of these cells were determined by dye exclusion assay

TKI	Target
Erlotinib	EGFR
AG1478	EFGR, PI4KA
Linsitinib	IGF1R, InsR
Imatinib	PDGFR, BCR-ABL, c-Kit
Alectinib	ALK, RET
Ceritinib	ALK

induced by alectinib or ceritinib treatment was next investigated. After treatment of the three GBM cell lines with the indicated dose of alectinib and ceritinib for 48 hours, cell death of these cells was assayed using the dye-exclusion method. As shown in Figure 4A, alectinib and ceritinib effectively induce cell death in a dose-dependent manner, and ceritinib shows higher cytotoxicity than alectinib in all three cell types. In addition, alectinib did not induce cell death in normal fibroblasts and astrocyte cells (data not shown). Next, to clarify what kind of cell death was induced in GBM cells by alectinib and ceritinib, the involvement of apoptosis, one of the representative programmed cell death machinery, was investigated. Lysates of the three GBM cells used in Figure 4A were analyzed by immunoblotting 48 hours after alectinib and ceritinib treatment. The cleavage of poly ADP-ribose polymerase (PARP), a well-established marker of ongoing apoptosis,[21] is observed in a dose-dependent manner of alectinib or ceritinib treatment in all three cell types with a few exceptions (Figure 4B). In addition, to further confirm the involvement of apoptosis in alectinib- or ceritinib-induced GBM cell death, U87MG and LN229 cells were treated with alectinib or ceritinib in the presence or absence of pan-caspase inhibitor zVAD-FMK (z-VAD) treatment

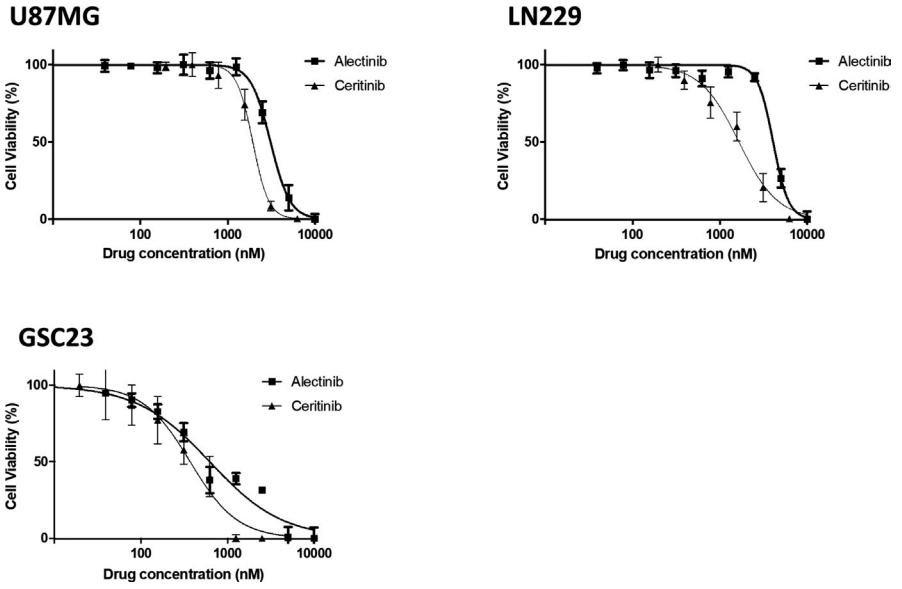
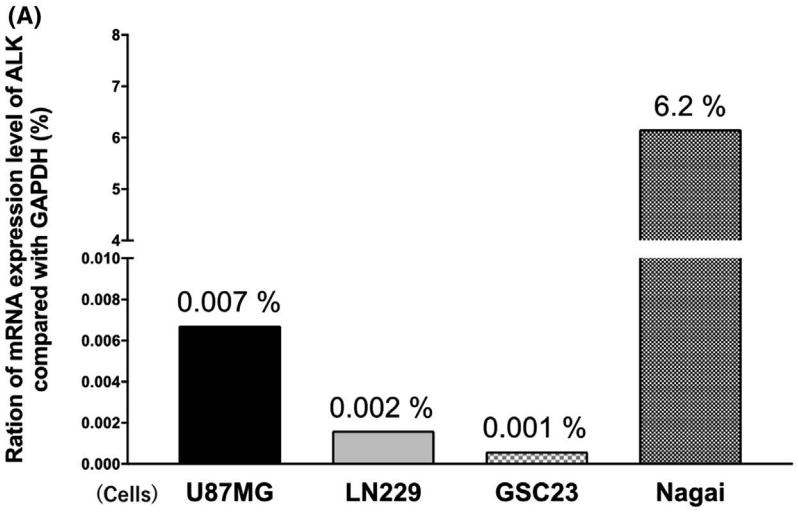


FIGURE 2 Alectinib and ceritinib inhibit the proliferation of human glioblastoma (GBM) cell lines. U87MG and LN229 cells (established human GBM cell lines) and GSC23 cells (patient-derived human GBM cell lines) were treated with vehicle (DMSO) or the indicated doses of alectinib or ceritinib. After 48 h, the relative ratio of viable cell numbers of the treated cells was determined by CCK-8 assay



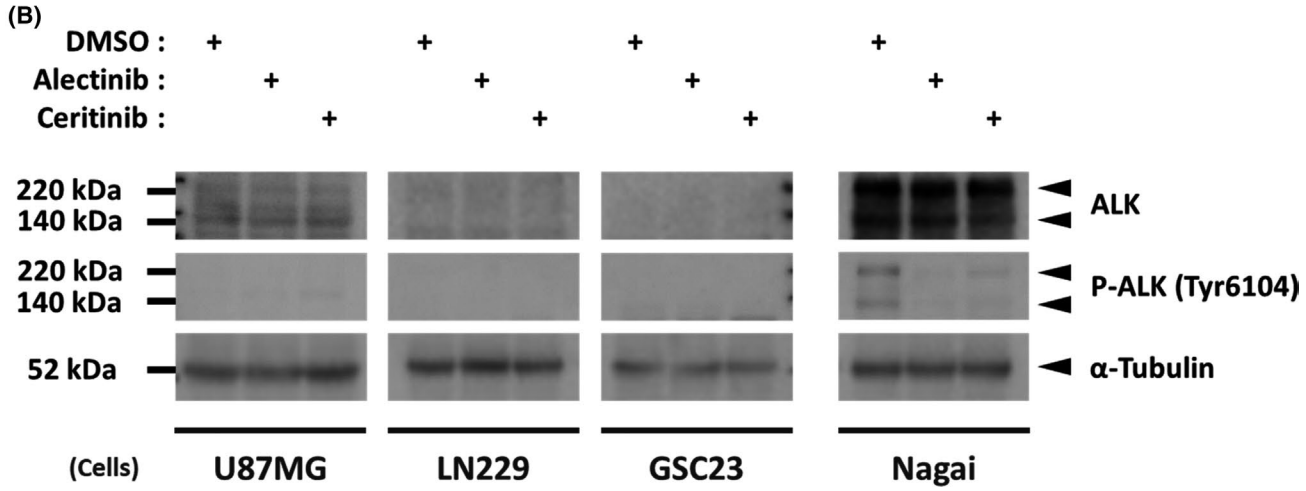


FIGURE 3 Expression levels of ALK in glioblastoma (GBM) cells are extremely low. A, ALK mRNA expression levels in the indicated GBM cell lines (U87MG, LN229, and GSC23) and Nagai cells, a human neuroblastoma cell line harboring amplified wild-type ALK, were determined by quantitative PCR analysis. GAPDH mRNA expression served as the loading control of RNAs. B, The indicated GBM cell lines and Nagai cells were treated with vehicle (DMSO), alectinib (3 μM), or ceritinib (3 μM). After 4 h, cell lysates were analyzed by immunoblotting using the indicated primary antibodies. Antibody against GAPDH was used to confirm the amount of protein loaded in each sample

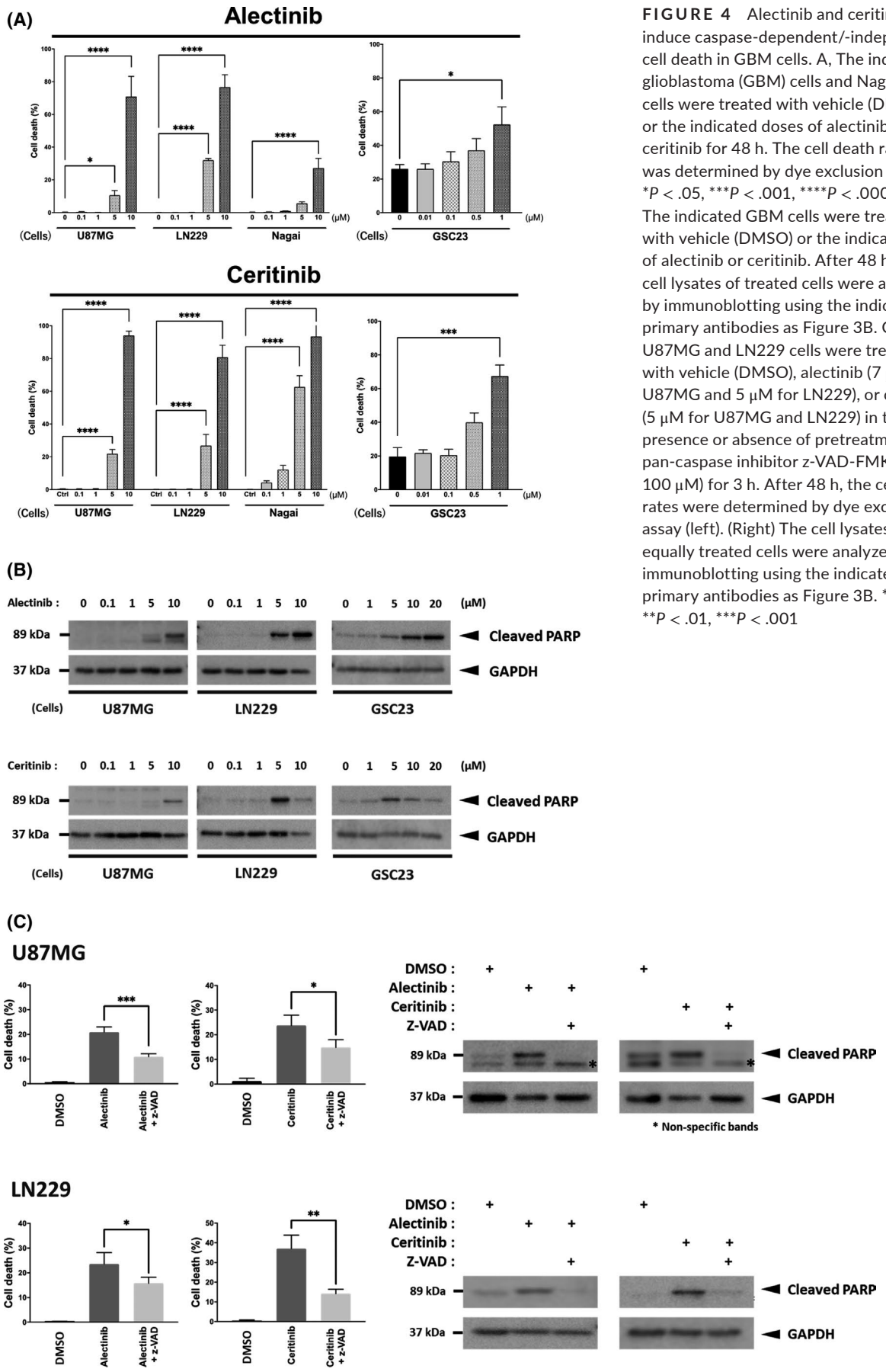


FIGURE 4 Alectinib and ceritinib induce caspase-dependent/-independent cell death in GBM cells. A, The indicated glioblastoma (GBM) cells and Nagai cells were treated with vehicle (DMSO) or the indicated doses of alectinib or ceritinib for 48 h. The cell death rate was determined by dye exclusion assay. $^{*}P < .05$, $^{***}P < .001$, $^{****}P < .0001$. B, The indicated GBM cells were treated with vehicle (DMSO) or the indicated dose of alectinib or ceritinib. After 48 h, the cell lysates of treated cells were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B. C, (Left) U87MG and LN229 cells were treated with vehicle (DMSO), alectinib (7 μM for U87MG and 5 μM for LN229), or ceritinib (5 μM for U87MG and LN229) in the presence or absence of pretreatment with pan-caspase inhibitor z-VAD-FMK (Z-VAD; 100 μM) for 3 h. After 48 h, the cell death rates were determined by dye exclusion assay (left). (Right) The cell lysates of equally treated cells were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

and subjected to cell death and immunoblotting assays. Whereas the cotreatment of alectinib or ceritinib with z-VAD almost completely suppressed cleavage of PARP induced by alectinib or ceritinib, it only partially blocked alectinib- or ceritinib-induced cell death in both cell lines (Figure 4C). Furthermore, in LN229 and GSC23 cells, treatment with higher concentrations of alectinib or ceritinib did not result in a further increase of PARP cleavage (Figure 4B). These results suggested that nonapoptotic cell death signaling, as well as caspase-dependent apoptosis cascades, might be activated in GBM cells treated with alectinib or ceritinib.

3.5 | Alectinib or ceritinib modulated the activation of various molecules downstream of RTK signaling and induced cell death mainly by suppressing STAT3 activation in human GBM cells

Because caspase-dependent/-independent GBM cell death triggered by alectinib and ceritinib was revealed, the molecular mechanism regulating this event was next investigated. On this occasion, it was hypothesized that alectinib and ceritinib might induce GBM cell death by other RTKs rather than their original target, ALK. Therefore, the representative downstream signaling pathways, which are common to RTKs, namely, STAT3, PI3K/Akt, and MAPK-ERK1/2–mediated pathways were investigated. In U87MG, LN229, and GSC23 cells, alectinib induced dose-dependent suppression of the tyrosine phosphorylation of STAT3 and activation of the tyrosine phosphorylation of janus kinase 2 (JAK2), which induces tyrosine phosphorylation of STAT3 (Figure 5A). In all GBM cells, ceritinib downregulated phosphorylation of Akt (Ser473) and ERK1/2 (Thr202/Tyr204), the activation barometer of each molecule, in addition to altered tyrosine phosphorylation of STAT3 and JAK2 (Figure 5B). Taken together, despite the lack of ALK activation, treatment with alectinib or ceritinib suppressed RTK downstream signaling in GBM cells.

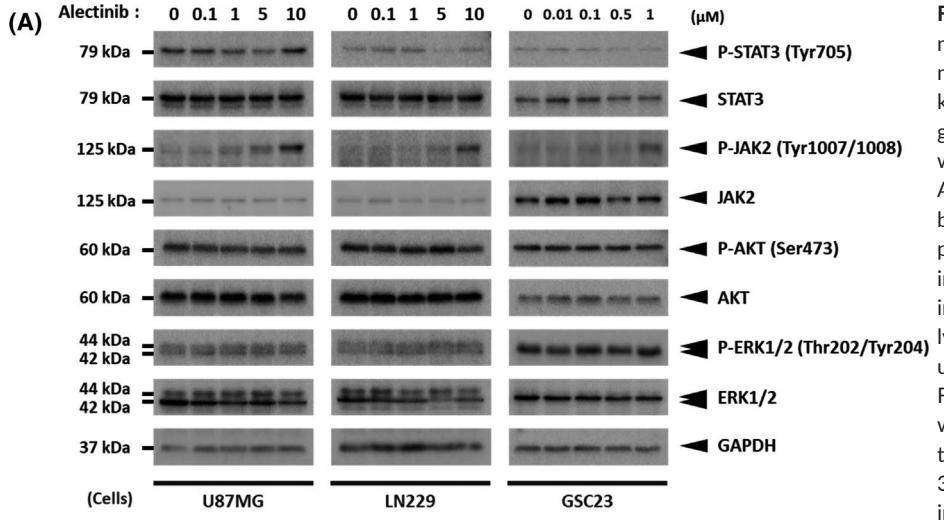
Moreover, the association between the above downstream signaling and cell death was also evaluated. The phosphorylation of STAT3, AKT, and ERK1/2 was independently inhibited by their specific inhibitors, stattic (a STAT3 inhibitor), LY-294002, and trametinib (Figure 5C). Treatment with 10 μM stattic exhibited high levels of cell death. In contrast, LY-294002 and trametinib did not induce similar amounts of cell death in U87MG cells compared with alectinib or ceritinib (Figure 5D). In addition, the concomitant treatment with ceritinib and various inhibitors did not demonstrate additional effects (Figure S1). Because stattic suppressed not only STAT3 but also AKT activation in U87MG cells (Figure S2), STAT3 knockdown was also performed using siRNA. As a result, STAT3 knockdown triggered cell death as well as cleavage of PARP in U87MG cells, suggesting induction of apoptotic cell death (Figure S3A and B). In addition, the concomitant treatment of siRNA with alectinib or ceritinib did not show additional cell-killing effects (Figure S3B). These results suggested that cell death induced by ceritinib originated from the suppression of RTK signaling pathways, especially STAT3 suppression.

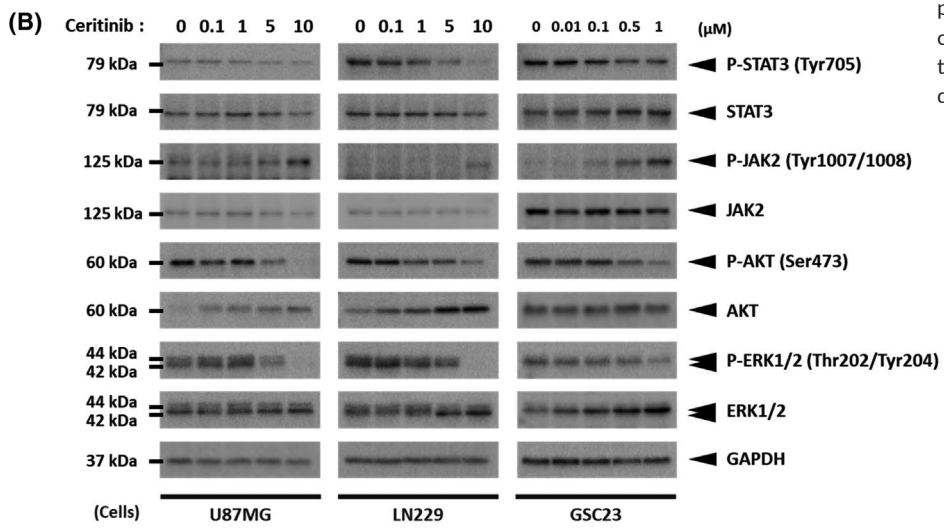
3.6 | ALK, IGF1R, or InsR knockdown by siRNA did not induce cell death

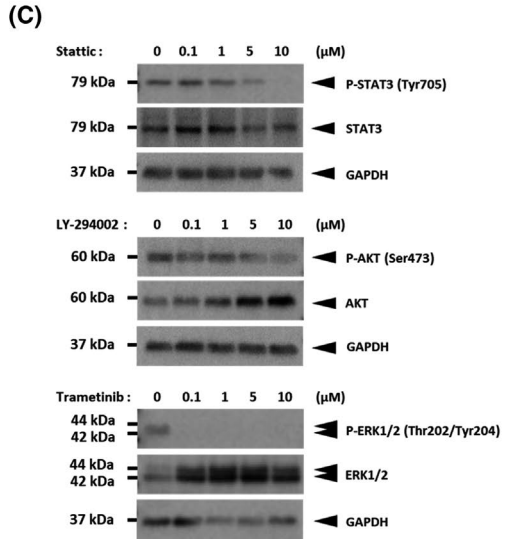
Because of the similarity of the kinase domain structure to each other, even single-targeted TKIs also block the activity of other tyrosine kinases when higher doses are used.[22-24] Therefore, it was hypothesized that alectinib or ceritinib also affected other tyrosine kinases, thereby inducing GBM cell death. Indeed, although the efficiency is approximately 80-fold lower than that of ALK, ceritinib also possesses enzymatic affinities and inhibitory effects on IGF1R and InsR, which belong to the insulin receptor superfamily, as well as ALK.[25] Alectinib also suppresses RTK rearranged during transfection (RET), although the expression and activation of RET were undetected in all GBM cell lines used in the study (data not shown). Therefore, whether IGF1R or InsR knockdown induces GBM cell death was tested. To determine this, IGF1R, InsR, and ALK were individually knocked down in U87MG cells, and the induction of cell death was assayed. As shown in Figure 6, each siRNA effectively suppresses the expression of IGF1R, InsR, or ALK, 48 hours after treatment with each siRNA. However, none of these siRNAs triggered U87MG cell death (Figure 6), suggesting that alectinib and ceritinib might induce GBM cell death independently of their known targets.

3.7 | Alectinib and ceritinib induced cell death in a TMZ-resistant GBM cell line

As mentioned in the introduction, the relapse of GBM cases by acquisition of resistance against current standard therapy is a major problem, and the identification of a novel therapy against these relapsed GBM cases is an urgent necessity. Based on the established results in this study, it was presumed that the off-target activity of alectinib or ceritinib might induce GBM cell death by inhibiting the activation of RTK downstream mediators. This cytotoxic mechanism is considered to be distinct from that of TMZ, an alkylating agent that is currently used for standard therapy of GBMs worldwide.[2] Thus, the antitumor effects of alectinib and ceritinib against GBMs with acquired TMZ resistance were investigated. The in vitro model of GBM cells with acquired TMZ resistance was established using U87MG cells (U87MG-R, Figure S4) based on the protocol we previously used with minor modification.[26] Alectinib or ceritinib (10 μM) effectively induced cell death in U87MG-R cells, whereas TMZ did not induce cell death in U87MG-R cells at a significantly high concentration (400 μM) (Figure 7). Also, alectinib and ceritinib suppressed STAT3 activity, and their effects against AKT and ERK1/2 activities in U87MG-R cells were similar to those of TMZ-sensitive control U87MG cells. In contrast, the treatment with TMZ did not affect STAT3, AKT, and ERK1/2 activities in U87MG-R cells (Figure S5). In addition, siRNA-mediated STAT3 knockdown triggered cell death as well as cleavage of caspase-9, one of the parameters of apoptosis cascade activation, in U87MG-R cells (Figure S6A and B). Furthermore, concomitant treatment of STAT3 siRNA with







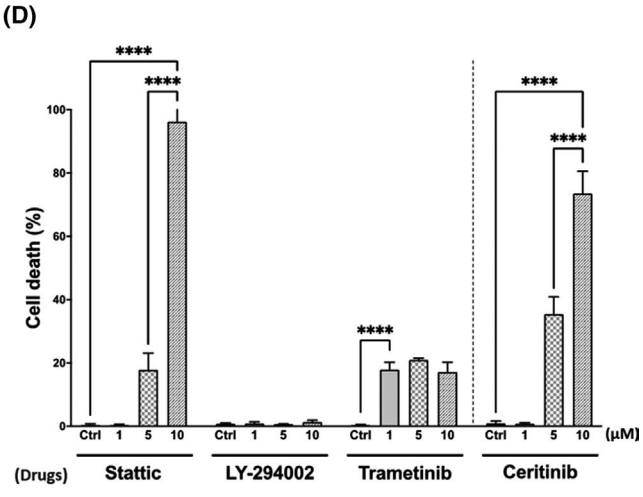


FIGURE 5 Alectinib and ceritinib modulate the activation of the various molecules downstream of receptor tyrosine kinase (RTK) signaling. A, The indicated glioblastoma (GBM) cells were treated with the indicated doses of alectinib. After 4 h, the cell lysates were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B. B, The indicated GBM cells were treated with the indicated doses of ceritinib. After 4 h, cell lysates were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B. C, U87MG cells were treated with the indicated doses of stattic (signal transducer and activator of transcription 3 [STAT3] inhibitor), LY29644 (PI3K inhibitor), or trametinib (MEK inhibitor). After 48 h, the cell lysates were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B. D, U87MG cells were treated as Figure 5C. After 48 h, the cell death rates were determined by dye exclusion assay

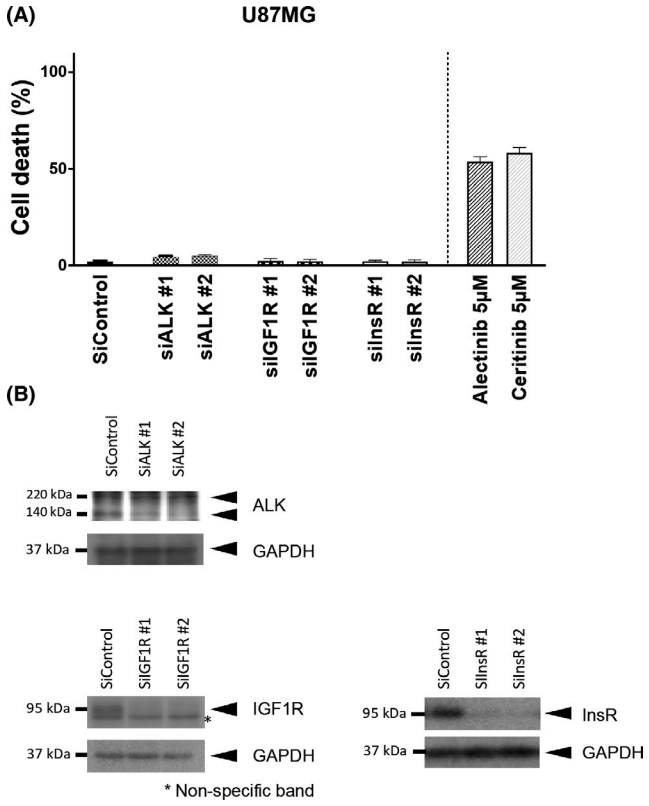


FIGURE 6 Knockdown of ALK, IGF1R, or InsR does not induce cell death against glioblastoma (GBM) cells. A, U87MG cells were transfected with the indicated siRNAs. After 48 h, the cell death rate was determined by dye exclusion assay. B, U87MG cells were treated as Figure 6A. After 48 h, the cell lysates were analyzed by immunoblotting using the indicated primary antibodies as Figure 3B

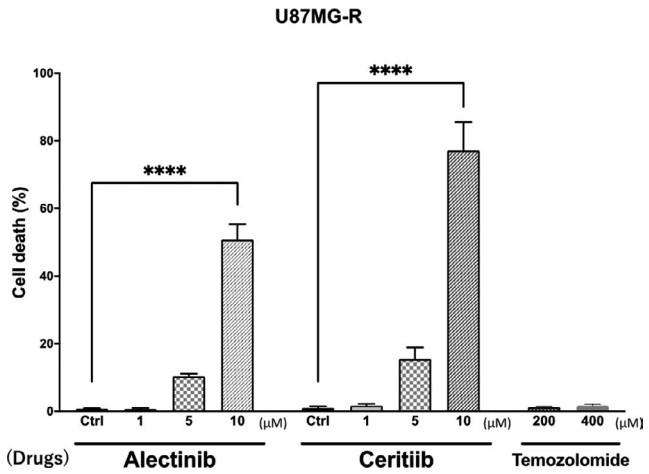


FIGURE 7 Alectinib and ceritinib effectively induce cell death even against glioblastoma (GBM) cells with acquired temozolomide (TMZ) resistance. U87MG cell clones with acquired TMZ resistance were treated with the indicated doses of alectinib, ceritinib, or TMZ. After 48 h, the cell death rates were determined by dye exclusion assay. ****$P < .0001$

alectinib or ceritinib did not demonstrate additional cell-killing effects (Figure S6B). These results suggest that alectinib and ceritinib are potential candidates as novel therapeutic agents against GBM cases that relapse after standard therapy.

3.8 | Orally administrated alectinib and ceritinib significantly prolonged the survival of mice with intracerebral glioma xenografts

Finally, whether alectinib and ceritinib exerted antitumor effects in animal GBM models was investigated using systemic administration. The intracranial GBM model using BALB/c nu/nu athymic mice was established by intracranial inoculation of U87MG or GSC23 cells, and control vehicle or the indicated dose of ALK inhibitors were orally administrated to these mice once a day for 14 consecutive days, as described in the Materials and Methods. Ceritinib (50 mg/kg) or alectinib (60 mg/kg) significantly prolonged the survival of mice harboring intracerebral U87MG (P-value = .038) or GSC23 (P-value = .026) xenografts, respectively (Figure 8). Also, immunohistochemical evaluation of the GBM xenograft of the mice used in Figure 8A demonstrated the suppression of STAT3 activity (phosphorylation) and the induction of PARP cleavage in the mice treated with ceritinib compared with the control mice treated with the vehicle (Figure 8C). Thus, the antitumor efficacy of alectinib and ceritinib in an animal brain tumor model was confirmed.

4 | DISCUSSION

ALK expression levels in GBM cell lines are generally very low,[18-20] and these reports are consistent with the current results. In addition, in the clinical database, ALK mutations in GBM cases are rare events that only occur in 1% of patients with GBM.[27] Therefore, the possibility of using ALK inhibitors as therapeutic agents for GBM has not been fully investigated. This study demonstrated their versatile potential for the first time.

Although the precise mechanism of cell death was not fully elucidated, alectinib and ceritinib suppressed the common RTK downstream signals, including STAT3 activation, independent of ALK activity and induced GBM cell death in this system. Because a previous report demonstrated that STAT3 is constitutively activated in GBM, whose inhibition suppresses proliferation and induces apoptosis of GBM cells,[28-30] the suppression of STAT3 activity plays a crucial role in alectinib- or ceritinib-induced GBM cell death.

As the sequence of the ATP-binding domain of RTKs is conserved to some extent,[22] TKIs often face difficulties retaining their specificities and selectivities,[23,24] and off-target TKI activities have been previously reported. One example is imatinib, which originally targets BCR-ABL, c-KIT, and PDGFR, and also

induces apoptosis in cells from patients with chronic lymphocytic leukemia, lacking its original targets in clinically achievable concentrations.[31] In addition, AMN107 (nilotinib) targeting BCR-ABL, c-KIT, and PDGFR arrests cell proliferation and induces apoptosis in B-lymphocytic cell lines lacking BCR-ABL, c-KIT, and PDGFR expression.[32] In line with this evidence, the off-target activity of crizotinib, a potent inhibitor of ALK and MET, has been previously reported; the (R)-enantiomer of crizotinib is a kinase inhibitor, whereas the major target of the (S)-enantiomer of crizotinib is the MutT homolog 1 enzyme,[33] which prevents the generation of point mutations caused by the incorporation of oxidized nucleotides into genomic DNA.[34-36] Even though neither alectinib nor ceritinib has recognized enantiomers exhibiting unexpected functions, further studies exploring the chemical characteristics or the metabolites of alectinib and ceritinib are warranted.

The comparably high IC_{50} values of alectinib and ceritinib also indicate their off-target activities against GBM cells. The determined IC_{50} values of alectinib and ceritinib for GBM cell lines were higher than those of other cell lines previously reported. For example, the IC_{50} values of alectinib for NSCLC cell lines harboring the ALK G1269A mutation and EML4-ALK fusion are 33.1 and 53 nM,

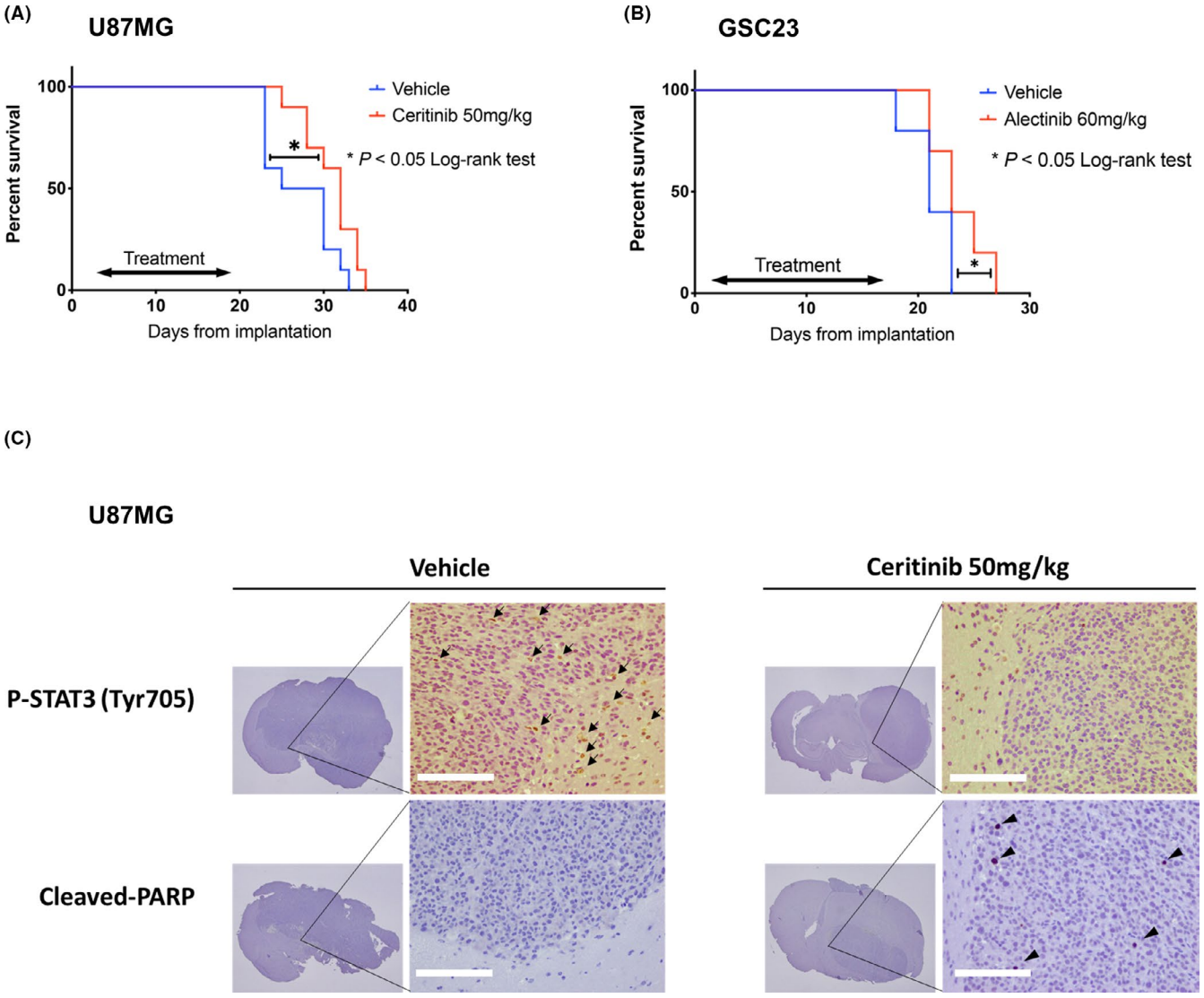


FIGURE 8 Oral administration of alectinib or ceritinib prolongs the survival of mice with intracerebral glioblastoma (GBM) xenografts. A, U87MG intracranial xenograft-bearing mice were treated with vehicle (control) or 50 mg/kg ceritinib for 14 consecutive days (days 4-17). Survival data of each treated mouse were presented in a Kaplan-Meier curve. *$P < .05$. B, GSC23 intracranial xenograft-bearing mice were treated with vehicle (control) or 60 mg/kg alectinib for 14 consecutive days (days 4-17). Survival data of each treated mouse were presented in a Kaplan-Meier curve. *$P < .05$. C, The representative immunohistochemical section images of the brain and intracranial GBM xenograft of the mice used in Figure 8A; the photos of the whole mouse brain (left images of each column) and GBM tissue section with higher magnification (right images of each column) stained with primary anti-phospho-STAT3 (P-STAT3 (Tyr705); upper images of each column) and anti-cleaved PARP (cleaved PARP; lower images of each column) antibodies are shown. The arrows indicate phospho-STAT3–positive cells, and the arrowheads indicate cleaved PARP-positive cells. Bar, 100 μm

respectively.[13,37] Also, past pharmacokinetic studies revealed that the maximal plasma concentrations of alectinib and ceritinib at clinically recommended doses reach 1.4 and 1.8 μM,[14,38] respectively. Compared with these previous results, at first glance, GBM cell lines require a considerably higher concentration of the ALK inhibitor to inhibit their proliferation in our present study. However, 1 μM of alectinib or ceritinib induced significant cell death against U87MG cells (data not shown) and patient-derived GBM cells GSC23 (Figure 4A) maintained by nutrient-reduced GBM sphere culture medium, which is considered more similar to the physiological condition compared with other generally used cell culture media supplemented with excess nutrients in our system. Therefore, alectinib and ceritinib are considered to have a possibility to exert efficient antitumor activity against GBM cells even by a lower dose if the GBM cells are grown under more physiological conditions.

It was noteworthy that alectinib and ceritinib exhibited antitumor activity against GBM cells with acquired resistance against TMZ. As the efficacy of concurrent and adjuvant treatment in adult GBM patients has been demonstrated since 2005, TMZ is the current, most widely applied chemotherapeutic agent in the first-line treatment for GBMs.[2] However, GBMs often gain resistance against TMZ after continuous treatment and relapse rapidly thereafter,[39] and there is no effective therapy against these relapsed GBMs. Consequently, recurrent GBMs acquire TMZ resistance, and GBM treatment becomes even more challenging. In this study, alectinib and ceritinib demonstrated antitumor potency in TMZ-resistant GBM cells. This result expands the possibility that ALK inhibitors are promising chemotherapeutic agents for patients with recurrent or TMZ-resistant GBM.

Lastly, in the in vivo study, treatment with 50 mg/kg ceritinib prolonged the survival of mice harboring intracerebral xenografts of U87MG cells, and 60 mg/kg alectinib demonstrated its efficacy against the xenografts of GSC23 cells with statistical significance. In this study, fixed doses of ALK inhibitors were administered for a limited period. A higher dose or longer period of ALK inhibitors might further prolong the survival of mice xenograft models. These in vivo results suggest that ALK inhibitors are promising therapeutic agents against GBM.

In conclusion, it was demonstrated that the second-generation ALK inhibitors, alectinib and ceritinib, were efficacious against GBM cells even without aberrant ALK expression. Although the precise mechanism was not fully determined, these results strongly suggested that alectinib and ceritinib induced GBM cell death by off-target activity. Additionally, they demonstrated their antitumor efficacy against TMZ-resistant GBM cells in vitro and in vivo in an intracranial GBM xenograft model. Thus, second-generation ALK inhibitors are promising and novel therapeutic agents for patients with GBM, including recurrent or TMZ-resistant GBM.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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