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Convection enhanced delivery of EGFR targeting antibody-drug conjugates Serclutamab talirine and Depatux-M in glioblastoma patient-derived xenografts

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Abstract

Background. EGFR targeting antibody-drug conjugates (ADCs) are highly effective against EGFR-amplified tumors, but poor distribution across the blood–brain barrier (BBB) limits their efficacy in glioblastoma (GBM) when administered systemically. We studied whether convection-enhanced delivery (CED) can be used to safely infuse ADCs into orthotopic patient-derived xenograft (PDX) models of EGFRvIII mutant GBM.

Methods. The efficacy of the EGFR-targeted ADCs depatuxizumab mafodotin (Depatux-M) and Serclutamab talirine (Ser-T) was evaluated *in vitro* and *in vivo*. CED was performed in nontumor and tumor-bearing mice. Immunostaining was used to evaluate ADC distribution, pharmacodynamic effects, and normal cell toxicity.

Results. Dose-finding studies in orthotopic GBM6 identified single infusion of 2 μg Ser-T and 60 μg Depatux-M as safe and effective associated with extended survival prolongation (>300 days and 95 days, respectively). However, with serial infusions every 21 days, four Ser-T doses controlled tumor growth but was associated with lethal toxicity approximately 7 days after the final infusion. Limiting dosing to two infusions in GBM108 provided profound median survival extension of over 200 days. In contrast, four Depatux-M CED doses were well tolerated and significantly extended survival in both GBM6 (158 days) and GBM108 (310 days). In a toxicity analysis, Ser-T resulted in a profound loss in NeuN+ cells and markedly elevated GFAP staining, while Depatux-M was associated only with modest elevation in GFAP staining.

Conclusion. CED of Depatux-M is well tolerated and results in extended survival in orthotopic GBM PDXs. In contrast, CED of Ser-T was associated with a much narrower therapeutic window.

Key Points

- CED of EGFR-targeting ADCs improves delivery and survival in GBM.
- Ser-T is unsuitable for treatment of GBM via CED infusion.
- Depatux-M is safe and effective when dosed with CED.

Importance of the Study

All GBM have tumor regions protected by a relatively intact blood–brain barrier (BBB) that can limit systemic delivery of otherwise highly effective therapeutic agents. We previously showed that poor distribution across the BBB limited the efficacy of the highly potent EGFR-targeted ADC Depatux-M. The current study demonstrates that limitations in delivery can be overcome by infusing ADCs by convection-enhanced delivery (CED). Multiple serial CED infusions of Depatux-M were well tolerated and associated with a marked prolongation in survival. In contrast, a related ADC with a more potent toxin, Ser-T, had a much narrower therapeutic window. The data provide continued enthusiasm for developing CED as a therapeutic delivery strategy for glioma-targeted ADCs. However, these data also highlight the importance of evaluating the risks for enhanced toxicity with CED and other clinical strategies being developed to enhance the delivery of therapeutics across the BBB.

Antibody-drug conjugates (ADCs) enable highly specific, intracellular delivery of potent cellular toxins and have significant antitumor efficacy in multiple malignancies. The tumor-specificity for an ADC is provided by antibody recognition and binding to an extracellular epitope that is unique or differentially expressed on tumor cells as compared to normal tissues. Highly potent toxin molecules are chemically linked to the antibody, and binding to an extracellular target and subsequent internalization and metabolism within the lysosome releases free toxin and results in cytotoxicity. In light of the tremendous clinical benefit of HER2-targeted ADCs in appropriately targeted malignancies,[1] there are significant efforts directed toward developing ADCs targeting the related epidermal growth factor receptor (EGFR). Approximately half of newly diagnosed glioblastoma (GBM) have high-level EGFR expression,[2] but poor delivery of a large, antibody-based therapeutic into regions of GBM with an intact blood–brain barrier (BBB) limits the efficacy of systemic therapy with these otherwise promising therapeutics.[3] The focus of this manuscript is to evaluate whether direct infusion of an ADC into these highly malignant brain tumors could provide significant survival gains in mouse models of GBM.

One of the most clinically advanced EGFR-targeted ADCs is depatuxizumab mafodotin (Depatux-M; ABT-414). The antibody backbone (ABT-806) was raised against a cryptic epitope within the juxta-membrane domain that is only exposed in the context of high-level EGFR signaling and in a variety of exon variants, such as deletion of exons 2-7 (EGFRvIII), commonly seen in EGFR-amplified GBM.[4] This antibody was conjugated to the microtubule toxin monomethyl auristatin F (MMAF) through a non-cleavable linker to develop Depatux-M. Building upon ABT-806, an affinity-matured antibody (AM-1) was conjugated to the highly potent DNA cross-linking agent talirine using a Cathepsin-B cleavable linker to develop serclutamab talirine (Ser-T, ABBV-321). The linker-toxin chemistry and physicochemical properties of the released toxin are key differences between these two ADCs; cysteine-maleimidocaproyl-MMAF (Cys-mcMMAF) released from Depatux-M is cell impermeant and can only kill tumor cells that have internalized the ADC, while talirine released from Ser-T is cell penetrant and can diffuse into adjacent non-EGFR-expressing cells to cause cytotoxicity. Given that normal CNS cells express low levels of EGFR, the risk profile for off-target normal cell toxicity via 'bystander killing' could be lower for Depatux-M.

Promising preclinical data in heterotopic patient-derived xenograft (PDX) GBM models, an anticipated lack of CNS toxicity, and early clinical trial data all supported further development of Depatux-M in GBM.[5] Two phase III randomized clinical trials in newly diagnosed and recurrent GBM (Intellance I and Intellance II, respectively) showed a trend toward activity, but failed to demonstrate a significant clinical benefit.[6,7] Further preclinical studies demonstrated that limited distribution across the BBB was a major factor limiting the efficacy of systemically administered Depatux-M and that artificial disruption of the BBB or direct, intratumoral injection of Depatux-M into orthotopic PDXs improved the efficacy of this agent.[3] Consistent with poor distribution contributing to limited efficacy in humans, a higher response rate and longer survival following systemic Depatux-M therapy was observed in GBM patients with smaller tumor volumes of <25 cm^3 in a recent analysis.[8] Based on all these data, this study evaluates the efficacy and toxicity of Depatux-M and Ser-T when delivered by convection-enhanced delivery (CED).

CED is a neurosurgical technique used to bypass the BBB and deliver novel therapies directly into brain tumors and adjacent brain tissues.[9] The technique involves placement of catheters within a target volume and then infusion of drug under positive pressure for an extended duration. Utilizing a pressure gradient, in addition to a concentration gradient, CED provides a large area of relatively uniform infusate distribution that can extend into peritumoral regions.[10] Key considerations for selection of therapeutics to infuse via CED include anti-tumor efficacy, residence time in tumor and normal tissue after infusion, and risk of normal tissue toxicity. In this context, both Depatux-M and Ser-T are highly selective for EGFRvIII mutant/EGFR-amplified tumor cells and are predicted to have an extended tissue half-life. Based on these considerations, the efficacy, toxicity, and pharmacodynamics of ADC CED infusions with Depatux-M and Ser-T were evaluated in two GBM PDXs. While both ADCs demonstrated significant antitumor efficacy, the much wider therapeutic window with Depatux-M supports further development of CED as a delivery strategy for this highly potent therapy for patients with recurrent, EGFRvIII mutant/EGFR-amplified GBM.

Materials and Methods

Antibodies and Reagents

An EGFR-specific antibody (ABT-806), isotype control antibody (AB095), control ADCs (AB095-MMAF and AB095-talirine), and EGFR-specific ADCs (Depatux-M and Ser-T) were provided by AbbVie and stored at –80°C in single-use aliquots. Other commercially available reagents are listed in Supplemental Methods.

In vitro Assays

In vitro cell culture of glioma cells and PDXs, cytotoxicity assays using CellTiter Glo, and Western blotting were performed as previously published.[3] *In vitro* immunofluorescence (IF) was performed as described previously.[11] Individual nuclei were delineated by DAPI staining, and each nucleus with ≥20 γH2AX foci was counted as positive.

Bystander cytotoxicity was assessed in F98 glioma cells transduced to express enhanced green fluorescent protein and firefly luciferase-2 (F98-eGFP/fLuc2) or EGFRvIII (F98EGFRvIII). Cells were seeded in different ratios (1:0, 4:1, 1:1, 1:4, 0:1) on 96-well flat bottom plates, treated with the indicated drugs, and incubated in an IncuCyte Live cell imaging system at 37°C, 5% CO_2 for 5 days. Percent cell confluence was measured using white light and green fluorescence microscopy. To check bystander effects in normal astrocytes (SVG-A), media was collected from GBM6 and GBM10 cultures treated for 4 days with either 10 μg/mL Depatux-M/AB095-MMAF or 0.1 μg/mL Ser-T/AB095-talirine and was added at a 1:1 ratio to existing media on exponentially growing SVG-A cells. After 7 days of incubation, cell proliferation was measured by CellTiterGlo assay.

In vivo Assays

Tissue sectioning, hematoxylin/eosin staining and immunohistochemistry (IHC) for NeuN was performed in the Mayo Clinic Pathology Research Core. For IF staining, mice with established orthotopic tumors were treated and euthanized followed by paraformaldehyde perfusion and processing as described previously.[3] All stained slides were scanned at 50x magnification on the LAS X DMI6000B (Leica, Buffalo Grove, IL). Quantification of stained slides was performed by a blinded technician using ImageJ Cell Counter. Drug quantification by matrix-assisted laser desorption ionization mass spectrometry imaging and tissue quantitation of Cys-mcMMAF by liquid chromatography were conducted as described in the Supplemental Methods.

Animal Studies

All animal studies were approved by the Mayo Institutional Animal Care and Use Committee. GBM PDX intracranial and flank tumor inoculation, flank tumor measurements and intracranial tumor bioluminescence imaging (BLI) were done as previously described.[3,12] For CED infusions, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. After skin disinfection, the prior incision from tumor implantation was re-opened. Mice were secured on a stereotactic stage with automated thermal support (Stoelting #53800M). The internal cannula (P1 #8IC315IS5SPC, cut 4mm projection) and guide cannula (P1 #8IC315GS5SPC, cut 3.5mm projection) were connected to PE tubing and secured with a single connector-assembly (P1 # C313C/SPC). The whole unit was secured vertically with a cannula holder (World Precision Instruments #505254). Drug solution was primed through the internal cannula, cannula tubing, and 22-gauge 25 μL Hamilton syringe (Hamilton # 80400) and the syringe was placed in the Legato 130 syringe pump (KD Scientific #788130). The cannula holder with attached internal cannula was lowered into the brain at the site of tumor inoculation until the plastic pedestal was flush with the mouse skull. A ramped infusion protocol was used with rate of infusion as follows: 3 μL at 0.2 μL/min, then 5 μL at 0.5 μL/min, and then 12 μL at 0.8 μL/min.[13] The needle and cannula were removed 10 min after completion of infusion.

Statistical Analysis

Cumulative survival probabilities were estimated using the Kaplan-Meier method. Survival comparisons across groups were calculated using the Log-Rank test. Analysis of variance or two sample *t*-test were used where appropriate. *P*-values <.05 were considered statistically significant.

Results

Ser-T efficacy in PDX cell culture

The *in vitro* sensitivity to Ser-T was evaluated across 4 EGFR mutant/EGFR-amplified GBM PDXs (GBM6, GBM39, GBM108, GBM12) and 2 EGFR non-amplified PDXs (GBM10, GBM43) and compared to previously published Depatux-M sensitivity (Supplementary Table 1). All four EGFR mutant/EGFR-amplified PDXs were highly sensitive to Ser-T with a 50% effective concentration (EC_{50}) ranging from 0.007 to 2 ng/ml, as compared to 90 and 1900 ng/ml for the non-amplified PDXs. In comparison to results with Depatux-M[3], Ser-T was notably more potent against GBM39 and GBM12.

Talirine toxin induces DNA inter-strand cross-links. The resulting DNA damage triggers phosphorylation of the variant H2 histone on Ser-139 (γH2AX), which was used to evaluate the time-course for induction of DNA damage by Ser-T. GBM6 and GBM108 cells were treated with 10 ng/ml Ser-T or AB095 and harvested 6, 24, and 48 hours later. The number of cells with elevated γH2AX foci (≥20 foci per nuclei) were quantitated. γH2AX levels began to increase with 24-hour exposure to drug and increased further at 48 hours in both PDX lines (GBM6, P = .009; GBM108, P = <.0001 at 24-hour time point; Figure 1A-B). Collectively, these data are consistent with significant, on-target cytotoxicity for Ser-T in EGFR mutant/EGFR-amplified GBM.

Potential for Bystander Killing by Ser-T

Intratumoral molecular heterogeneity is a defining feature of GBM, and most EGFR-amplified GBM harbor a subset of tumor cells with low-level EGFR expression.[14] To evaluate the potential for bystander killing, the cytotoxicity of Ser-T was evaluated in F98 glioma cell line with low endogenous levels of EGFR expressing an eGFP-fLuc2 construct (F98-eGFP/fLuc2) or a human EGFRvIII construct (F98-EGFRvIII, Supplementary Figure 1A). Using green-fluorescent cell confluence to specifically evaluate cytotoxicity of the GFP+/non-EGFR expressing cells, Ser-T was specifically toxic to the F98-eGFP/fLuc2 cells only when co-cultured with F98-EGFRvIII cells at ratios of 80%, 50%, and 20% (Figure 1C).

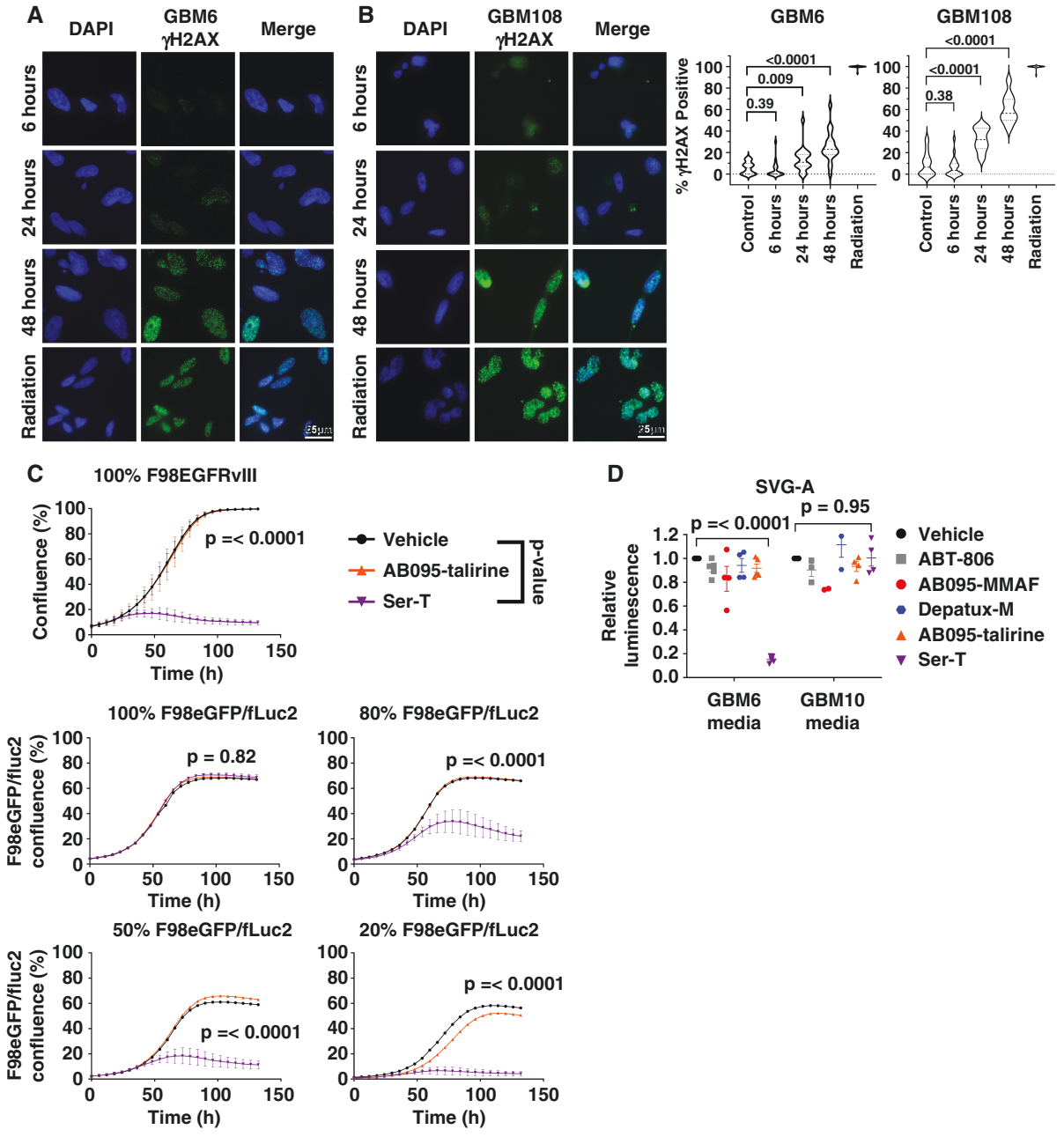


Figure 1 **In vitro cytotoxicity of Ser-T.** (**A-B**) GBM6 or GBM108 cells were incubated with 10 ng/ml Ser-T and harvested after 6, 24, and 48 hours. Cells were treated with 5 Gy radiation and harvested after 30 min as a positive control. Quantitation from three independent studies is plotted. (**C**) Bystander cytotoxicity of 50 ng/ml* Ser-T or non-targeted AB095-talirine was measured as percent confluence using white light or green fluorescence. (**D**) Conditioned media collected from GBM6 or GBM10 cells treated with the indicated drugs were added 1:1 to existing media of SVG-A cultures and cytotoxicity assessed seven days later by CellTiterGlo. All studies were repeated three times and representative results are shown. *One study performed at 30 ng/ml.

Consistent with talirine-mediated bystander toxicity, γH2AX foci were identified in both F98-EGFRvIII and F98-eGFP/fLuc2 cells when co-cultured (Supplementary Figure 1B). Because F98 cells are insensitive to auristatins, bystander cytotoxicity was not tested with Depatux-M.

The potential for Ser-T or Depatux-M-mediated bystander toxicity to normal cells was evaluated in SVG-A human astrocytes. Because SVG-A cells and glioma cells have markedly different growth rates, bystander killing was evaluated using a conditioned media strategy. Without conditioned media, the non-EGFR expressing SVG-A and GBM10 cells were resistant to 1000 ng/ml Ser-T and Depatux-M. Of note, SVG-A cells are highly sensitive to the related, free auristatin toxin MMAE (data not shown). EGFRvIII mutant/EGFR amplified GBM6 cells were highly sensitive to both ADCs (Supplementary Figure 1C). Conditioned media was collected from GBM6 and GBM10 cultures after a 4-day incubation with relevant drugs and added 1:1 to existing media on exponentially growing SVGA cell cultures. Subsequent growth of SVG-A cultures was significantly suppressed with conditioned media collected from Ser-T-treated GBM6 cells as compared to vehicle, AB095-MMAF, Depatux-M or AB095-talirine conditioned media ($P < .0001$, vehicle). In contrast, conditioned media collected from non-EGFR amplified GBM10 treated with any of the ADCs did not significantly impact the growth of SVG-A cultures ($P = .95$; Figure 1D). Collectively, these data demonstrate the potential for bystander cytotoxicity mediated by Ser-T, and not Depatux-M, for both tumor cells and astrocytes.

Manipulation of BBB Increases Efficacy and Drug Delivery

The efficacy of Ser-T was tested in heterotopic tumors to assess tumor cell autonomous drug sensitivity without limitations in drug delivery imposed by the BBB. In four EGFR mutant/EGFR-amplified PDX models, Ser-T (0.1 mg/kg, intraperitoneal injection) provided significant survival benefits (Supplementary Table 2). Weekly intraperitoneal dosing with Ser-T extended the median survival, compared to AB095 control, in GBM26 (Figure 2A; EGFR amp with A289T mutation; $P = .003$, 387 days vs 67 days, respectively) and GBM59 (Figure 2B; EGFRvIII/EGFR amp; $P = .0001$, 49 days vs 25 days, respectively). Even with a limited number of Ser-T doses, significant survival gains were observed in both GBM08 (Figure 2C; EGFR wild-type, 2 doses, $P = .005$, 113 days vs 60 days, respectively) and GBM39 (Figure 2D; EGFRvIII/EGFR amp, 4 doses, $P = .002$, 179 days vs 34 days, respectively). However, consistent with EGFR-specific toxicity, Ser-T provided no survival

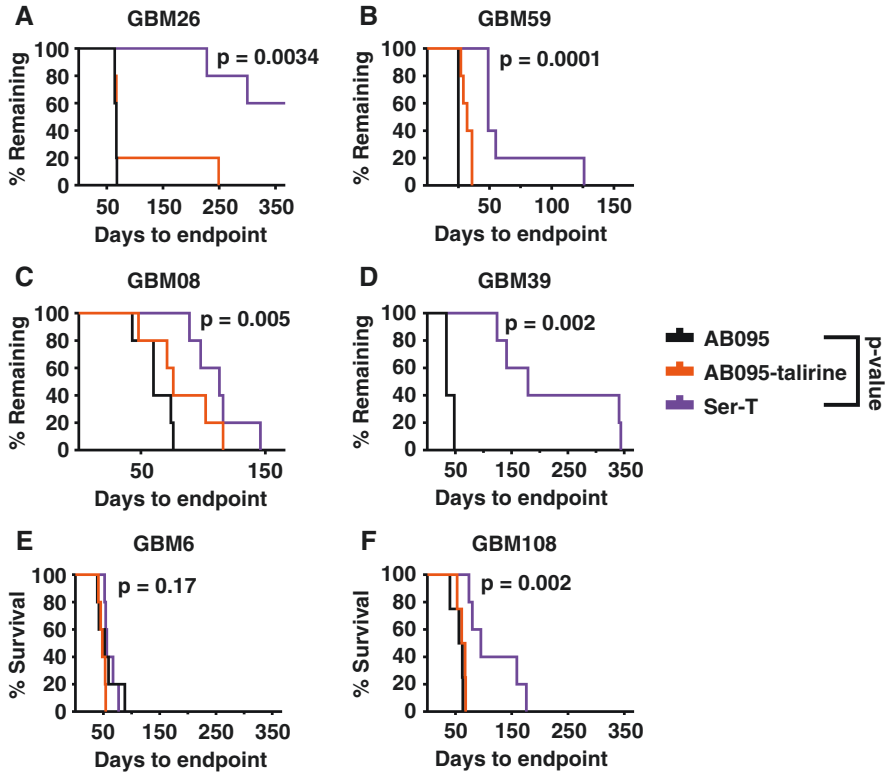


Figure 2 **Efficacy of Ser-T in GBM PDXs following systemic administration.** Heterotopic EGFR mutant/EGFR-amplified PDXs were treated systemically with the indicated agents with (**A-B**) continuous weekly dosing, (**C**) for 2 weeks, or (**D**) 4 weeks. Orthotopic EGFRvIII mutant/EGFR-amplified PDXs (**E**) GBM6 and (**F**) GBM108 were treated systemically with the indicated agents with continuous weekly dosing. Time for mice to exceed their endpoint is plotted and differences between indicated groups assessed by Log-Rank test.

benefit in two EGFR non-amplified PDXs GBM10 and GBM22 (Supplementary Figure 2A). Overall, response to Ser-T was specifically robust in multiple EGFR mutant/EGFR-amplified heterotopic GBM PDXs.

The efficacy of Ser-T was evaluated in two EGFRvIII mutant/EGFR-amplified GBM orthotopic PDX models. In a previous study, the related Depatux-M was highly effective with systemic dosing in both GBM6 and GBM108 grown as heterotopic tumors, but much less effective when grown as orthotopic tumors.[3] Similarly, systemically administered Ser-T (0.1 mg/kg weekly until moribund) was ineffective in orthotopic GBM6 without significant survival extension (P = .17; Figure 2E). In GBM108, Ser-T treatment extended median survival to 96 days as compared to AB095 (60 days) or AB095-talirine (65 days) treatment (P = .002; Figure 2F). In GBM108, robust disruption of the BBB through stable expression of vascular endothelial growth factor-A[15] markedly increased the efficacy of Ser-T with a median survival of 286 days compared to 23 days for AB095 control (P = .0009; Supplementary Figure 2B). These data are all consistent with previous Depatux-M data suggesting the efficacy of systemically administered EGFR-targeted ADCs can be limited by poor distribution into orthotopic tumors.[3]

CED-mediated Intra-tumoral Delivery of ADC

CED is an established, clinically relevant strategy to deliver novel therapeutics into brain tumors regardless of the integrity of the BBB. The distribution of AB095 or Ser-T was evaluated in mice with established orthotopic GBM6-eGFP/fLuc2 tumors. Groups were infused via CED with Ser-T and harvested 48, 96, or 120 hours later or infused with AB095 control antibody and harvested 48 hours later (n = 3). Fluorescence microscopy verifies infusion parameters by demonstrating extensive distribution of ADC throughout the tumor volume and much of the ipsilateral hemisphere (Figure 3A). Consistent with toxin-mediated cytotoxicity, co-IF staining of serial brain sections demonstrates robust induction of γH2AX in tumors infused with Ser-T, as compared to AB095 (Figure 3B). Collectively, these data provide direct evidence that CED in orthotopic GBM tumors can deliver pharmacologically effective concentrations of ADCs to a tumor volume and the surrounding 'normal brain' that is commonly infiltrated by GBM cells.

Pilot Efficacy Evaluation of ADC CED Infusion in Orthotopic Tumors

A pilot study was performed in orthotopic tumors to define a tolerable and effective infusion dose for Ser-T. Mice were implanted with GBM6-eGFP/fLuc2 and treated with AB095 or four doses of Ser-T (n = 3/dose level). The impact of ADC infusion on tumor growth was evaluated by BLI and impact on survival was measured as time to reach a moribund state. Based on BLI, each of the four Ser-T doses resulted in tumor regression (Supplementary Figure 2C), but only the intermediate Ser-T dose (2 μg) provided robust survival extension (Figure 3C). While the limited survival benefit at higher doses was not directly explored in this experiment, subsequent studies suggest this is could be related to enhanced toxicity. Based on these results, a dose of 2 μg Ser-T was selected for subsequent studies.

CED infusion of Depatux-M was explored in a similar study design. Mice with orthotopic GBM6-eGFP/fLuc2 were treated with AB095 or four dose levels of Depatux-M (n = 3). Interestingly, all Depatux-M dose levels tested were well tolerated, resulted in reduction in BLI signal (Supplementary Figure 2D), and significantly extended survival (Figure 3D). Based on these data, an intermediate dose of 60 μg was selected for subsequent studies.

Serial CED Infusions of ADCs in Orthotopic PDXs

The efficacy of Ser-T and Depatux-M CED infusions were compared in orthotopic GBM6- and GBM108-eGFP/fLuc2 PDXs. Envisioning a clinical strategy of repeated CED infusions over several months, we evaluated the efficacy of ADC CED performed every 21 days for four cycles. Mice with orthotopic tumors were treated with (i) AB095, (ii) AB095-talirine, (iii) Ser-T, (iv) AB095-MMAF, and (v) Depatux-M (n = 5). CED provided robust and sustained distribution of ADCs (Supplementary Figure 3A-B). In comparison to AB095, both AB095-talirine and Ser-T treatment significantly reduced BLI signals in GBM6 (P = .005 and .003, respectively; Supplementary Figure 2E). However, all mice treated with AB095-talirine died prior to the planned fourth infusion, and four of five Ser-T-treated animals died shortly after the fourth infusion (Figure 4A). Consistent with significant anti-tumor activity coupled with normal tissue injury, histologic evaluation of brains from euthanized mice treated with either AB095-talirine or Ser-T are notable for minimal tumor present coupled with significant normal tissue necrosis in the right anterior hemisphere in the region of CED infusion (Supplementary Figure 4A). Thus, despite good tolerability after a single infusion, four serial infusions of Ser-T at the same dose-level were poorly tolerated.

In contrast, MMAF-containing ADCs were well tolerated and highly effective in GBM6. Four serial CED infusions of AB095-MMAF were associated with significant suppression of tumor growth (Supplementary Figure 2E) and significantly extended median survival (120 days) compared to AB095 (53 days). Treatment with Depatux-M was associated with even greater median survival (158 days, P = .002, Figure 4B). Notably, moribund mice had evidence of recurrent tumor and no evidence of brain necrosis on histologic sections (Supplementary Figure 4A). Thus, despite a much higher infusion dose, Depatux-M was more effective and better tolerated than Ser-T in GBM6.

A second comparison was performed in GBM108. Based on the results with GBM6, Ser-T and AB095-talirine dosing was maintained at 2 μg/infusion but limited to two CED infusions 21 days apart, while four infusions were given for AB095, AB095-MMAF and Depatux-M. With the two-infusion regimen, median survival was extended with Ser-T (294 days) treatment as compared to AB095 control (57 days) (P = .057; Figure 4C, Supplementary Figure 2F), and brains from moribund mice demonstrated recurrent tumor without evidence of brain necrosis (Supplementary Figure 4B). Depatux-M was also highly effective with a median survival of 310 days (P = .02; Figure 4D). Although

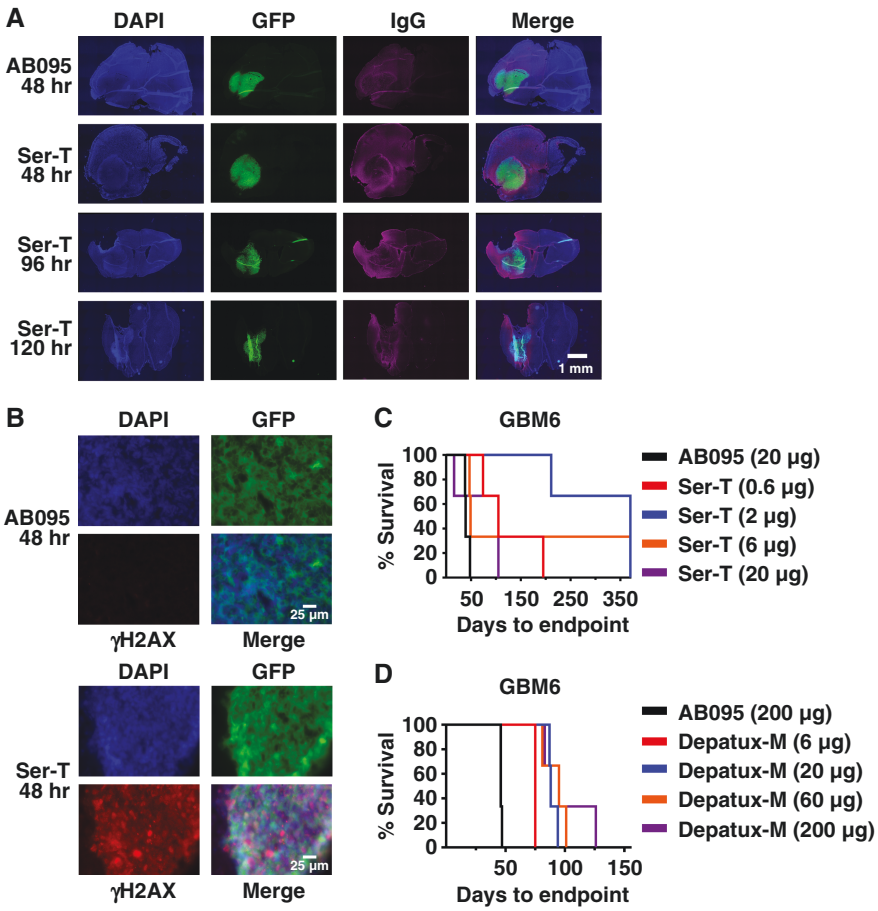


Figure 3 **CED brain infusion of orthotopic PDXs. (A)** Distribution of human IgG in mice with orthotopic GBM6-eGFP/fLuc2 following CED with AB095 or Ser-T was assessed by IF microscopy 48, 96, and 120 hours after infusion. **(B)** Immunohistochemistry for γH2AX in GBM6 tumors treated via CED with 6 µg AB095 or Ser-T and harvested 48 hours later. (**C**) Kaplan-Meier survival for mice with GBM6-eGFP/fLuc2 orthotopic tumors treated with a single CED infusion of AB095 or escalating doses of Ser-T. (**D**) Similar study performed with AB095 or Depatux-M ($n = 3$/group). Arrow indicates time of dosing. Images in A and B are representative of three mice/group.

not statistically significantly different from placebo, the median survivals following treatment with AB095-talirine (230 days) and AB095-MMAF (130 days) are notable. These data demonstrate the potent anti-tumor activities of both MMAF and talirine-containing ADCs but also highlight the potential for loss of EGFR specificity when infused by CED.

Talirine-ADCs are Toxic to Normal CNS Cells

The toxicity profile for CED infusions from Depatux-M or Ser-T were investigated further. To evaluate non-EGFR specific toxicities, tumor-naive mice were treated with AB095 (228 µg), Depatux-M (6, 60, 200 µg) or Ser-T (0.06, 0.6, 6 and 20 µg) (n = 3/dose level). Mice were euthanized 7 days later and brains processed for NeuN IHC as a marker of mature neurons. At this timepoint, CED of 20 µg Ser-T into normal brain resulted in a marked decrease in NeuN-positive cells in the ipsilateral hemisphere (492 ± 192 cells per HPF) as compared to lower Ser-T doses or AB095 control (917 ± 94 cells per HPF) ($P = .03$; Figure 5A-5B, Supplementary Figure 5A). In contrast, none of the Depatux-M doses had an impact on NeuN+ cell density (960 ± 31 cells per HPF with 200 µg Depatux-M; $P = .49$). Reactive astrocytes are another potential marker for CNS toxicity and can be readily evaluated by immunostaining for glial fibrillary acidic protein (GFAP). Consistent with the observed differential in neuronal toxicity, GFAP staining was markedly elevated in animals infused with 20 µg Ser-T as compared to no change following 200 µg Depatux-M infusion (Supplementary Figure 5B). Serial sections were also stained for γH2AX to detect DNA damage signaling induced by the talirine toxin. As seen in Figure 5C-D, infusion with 20 µg Ser-T was associated with significantly elevated γH2AX compared to AB095 or Depatux-M ($P < .0001$; Figure 5C-D). Moreover, γH2AX co-localized with both NeuN+ (Figure 5E) and GFAP+ cells following Ser-T CED (Supplementary Figure 5C). Collectively, these data are all consistent with increased risk of normal tissue injury associated with Ser-T CED infusion, which has a much narrower therapeutic window than Depatux-M.

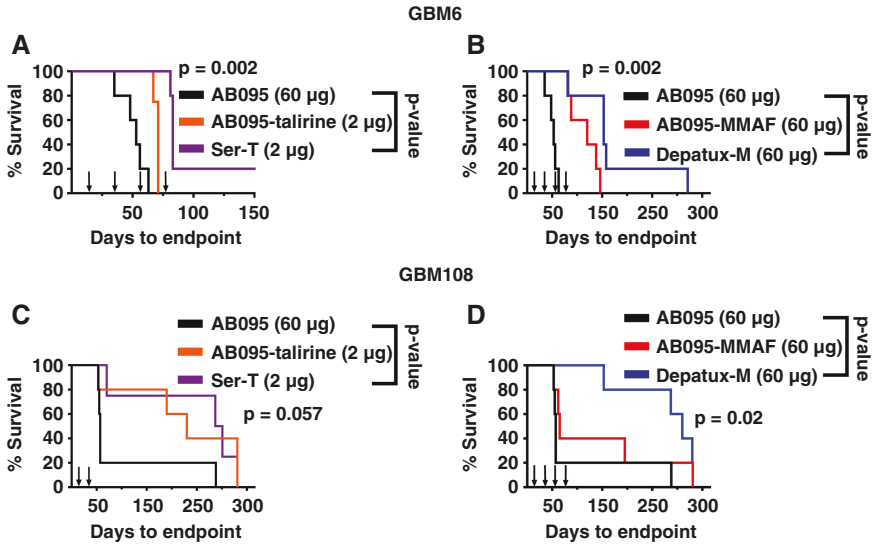


Figure 4 **Comparison of Ser-T and Depatux-M in orthotopic PDXs.** (**A-B**) Mice with established orthotopic GBM6-eGFP/fLuc2 were randomized into five groups (n = 4 or 5/group) and treated with the indicated drugs delivered by four CED infusions (arrows) spaced 21 days apart. The results for Ser-T and Depatux-M are plotted separately for clarity. (**C-D**) Similar studies were performed with GBM108-eGFP/fLuc2 tumors (n = 5/group), but dosing of AB095-talirine or Ser-T were limited to two infusions. Comparison between indicated groups were assessed using Log-Rank test.

Tissue Distribution of Released Toxin Released from Depatux-M

Levels of the released toxin were evaluated by mass spectrometry following CED or systemic dosing of Depatux-M. At 24 hours after Depatux-M (20 µg) infusion into the anterior right brain, animals were euthanized and released Cys-mcMMAF was measured in the four brain quadrants. The highest measured levels of released Cys-mcMMAF was in the right anterior brain and above the limit of quantitation (<LOQ) in four of five mice (44.35, 1.77, 2.95, and 1.18 ng/g), while levels in the left-brain quadrants were above the LOQ in only two mice (6.79 and 1.31 ng/g, Supplementary Table 3). In a second cohort of mice euthanized 96 hours after CED, released Cys-mcMMAF was nominally higher than the LOQ for all samples. Plasma levels from all mice infused by CED were below the LOQ. Similarly, brain and plasma levels of Cys-mcMMAF were <LOQ for mice dosed systemically with Depatux-M (5 mg/kg IP) and collected 24 and 96 hours later (data not shown).

Geographic distribution of released Cys-mcMMAF following internalization and intracellular cleavage of MMAF-containing ADCs was measured by MALDI-MSI. Mice with orthotopic GBM6-eGFP/fLuc2 were treated with Depatux-M or controls by systemic (IP) or direct (CED) dosing (n = 3/group) and then euthanized 48 hours later. Analysis by MALDI-MSI for released Cys-mcMMAF toxin demonstrated significantly elevated levels in tumor regions (9,417.7 ± 7,353.3 ng/g, n = 3) but levels <LOQ in surrounding "normal brain" (Figure 6A-B, Supplementary Figure 5D). Furthermore, Cys-mcMMAF tumor concentrations of all IP dosed animals but one (1,249 ng/g) were <LOQ. Collectively, these data suggest EGFR-targeting Depatux-M is a highly effective and safe therapeutic for EGFRvIII mutant/EGFR-amplified GBM, but that efficacy might be limited by poor drug delivery across an intact BBB which can be overcome by CED.

Discussion

EGFR genomic amplification promotes glioma progression, invasion and cell survival, and high-level EGFR expression on the surface of tumor cells can demarcate EGFR-amplified GBM from other cells within the adult central nervous system. Despite pre-clinical studies suggesting suppression of EGFR signaling results in prolonged survival,[16] numerous clinical trials have failed to show efficacy of small molecule EGFR kinase inhibitors in GBM. Failure is likely related to limited drug delivery across the BBB, intra-tumoral heterogeneity of EGFR expression, compensatory signaling in the face of EGFR suppression, and dose-limiting gut and skin toxicities.[8,17] In contrast, the EGFR-targeted ADCs tested here capitalize on a unique oncogenic EGFR epitope for tumor-specific delivery of highly potent toxins,[18] and dose-limiting gut and skin toxicities are uncommon following systemic administration of these drugs. Our previously published study[3] and the current manuscript both highlight the potential for profound survival benefit from EGFR-targeted ADCs when adequately delivered across the BBB and provide a strong rationale to pursue these or other ADCs as potential clinically important novel therapies for GBM.

Multiple strategies are being developed to improve drug delivery into brain tumors. The most clinically advanced include direct infusion by CED, intravascular drug delivery coupled with BBB disruption using osmotic

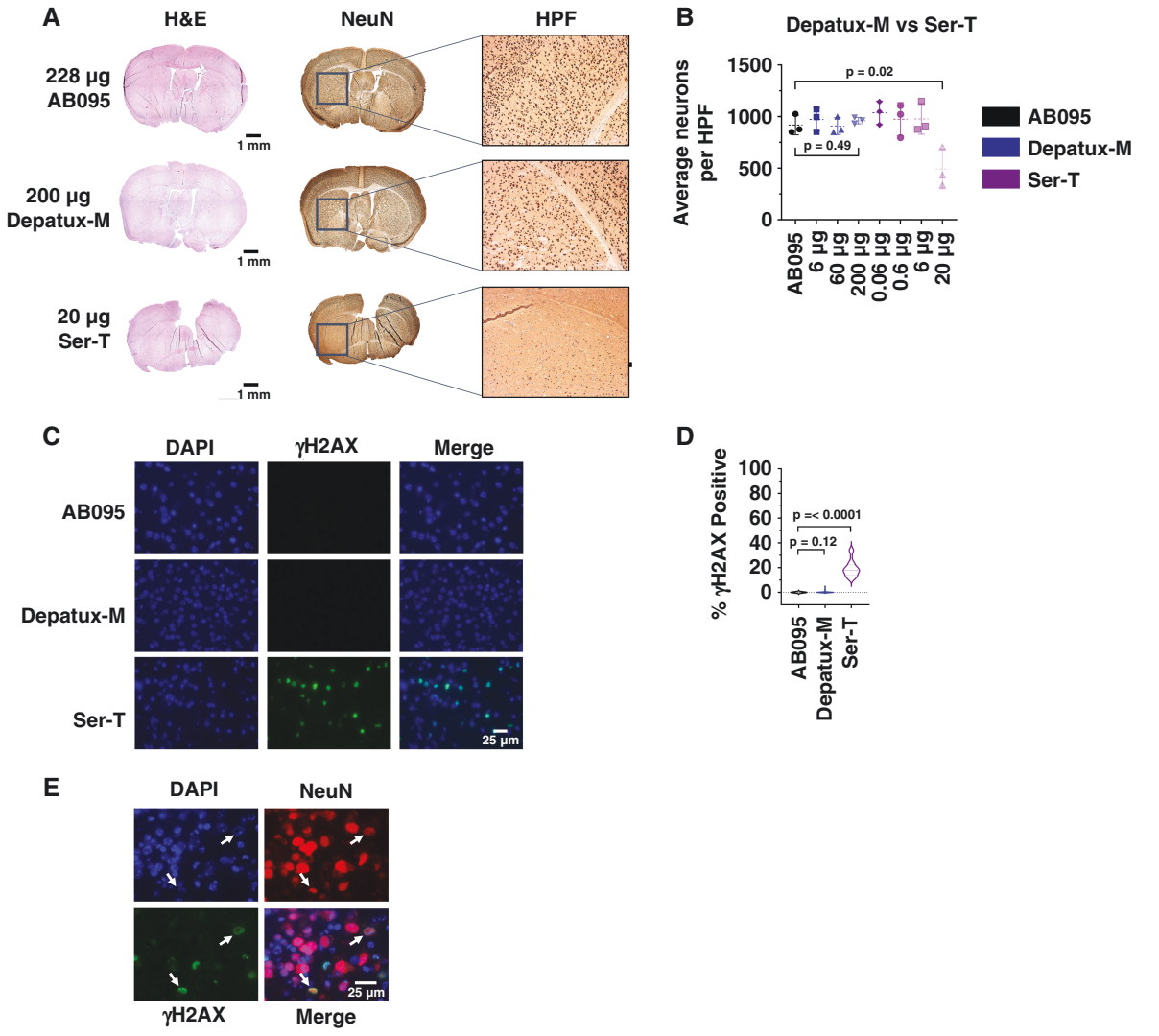
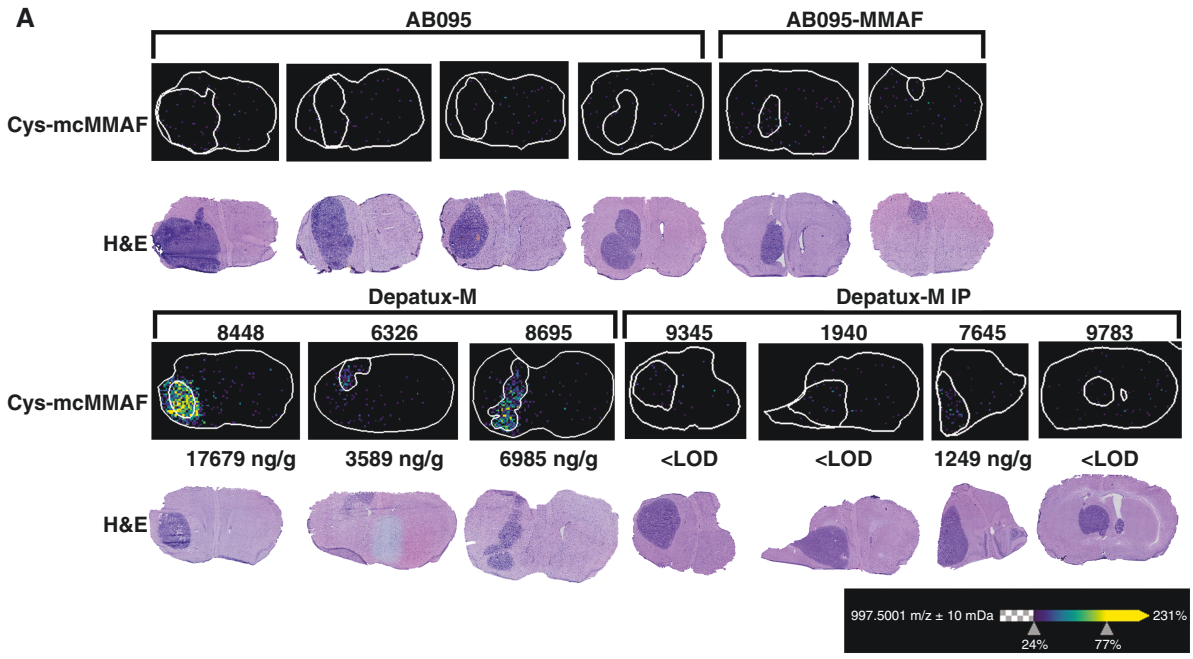


Figure 5 **Evaluation of central nervous system toxicity** Cohorts of non-tumor bearing mice ($n = 3$/group) were infused by CED with a range of doses for AB095, Depatux-M, or Ser-T and euthanized seven days later and processed for histology. (**A**) Sections were stained for H&E and NeuN staining. (**B**) Average number of neurons per HPF is quantified across all mice treated. Tissue sections from mice treated as above were processed for IF staining for (**C**) γH2AX. (**D**) The fraction of cells with nuclear γH2AX staining was quantitated. (**E**) Co-IF of γH2AX co-localization with NeuN+ cells evaluated following Ser-T treatment. For each photomicrograph, representative images are shown for n=3 mice per condition.

agents, or focused ultrasound. While each strategy has been used to enhance delivery of ADCs or other large biological molecules,[19,20] the transient nature of BBB disruption with the latter techniques may limit the level of enhanced ADC accumulation within tumor regions. In contrast, CED infusion of ADCs provides robust and sustained distribution throughout the tumor and surrounding normal brain tissue in our PDX models (Figure 3A, 6). Despite early challenges with CED,[21] improvements in catheter design and catheter placement have been pivotal for numerous recent clinical trials using CED to infuse other biologic molecules including immuno-toxin conjugates, viruses and nanoparticle therapeutics.[9,22] Thus, CED is a viable clinical strategy for enhancing the delivery of ADCs into GBM tumors.

The results from this study highlight several important characteristics of ADCs that may be important considerations for a future clinical trial. While both ADCs provided substantial improvement in survival, Ser-T was associated with substantially greater normal tissue toxicity. This is especially notable since both the EGFR-targeted and non-targeted talirine ADCs resulted in brain necrosis after repeated CED infusions. These data are consistent with accumulation of talirine-induced DNA damage-associated γH2AX foci in normal tissues, astrocytes (GFAP+) and neurons (NeuN+) in an EGFR-independent manner. Unlike microtubule-targeted agents such as MMAF, talirine has significant cytotoxic effects against both proliferating and quiescent cells, which may contribute to the significant CNS toxicity observed with Ser-T.[23] Although a different



B

Treatment	Sample	Adjusted concentration (ng/g)
Depatux-M CED	8448 normal	<LOD
	8448 tumor	17 679
	6326 normal	<LOD
	6326 tumor	3589
	8695 normal	<LOD
	8695 tumor	6985
Depatux-M IP	9345 normal	<LOD
	9345 tumor	<LOD
	1940 normal	<LOD
	1940 tumor	<LOD
	7645 normal	<LOD
	7645 tumor	1249
	9783 normal	<LOD
	9783 tumor	<LOD

Figure 6 **MALDI-MSI evaluation of Cys-mcMMAF.** (**A**) Mice with established GBM6-eGFP/fLuc2 tumors were dosed once as indicated, with AB095 (60 μg CED), AB095-MMAF (60 μg CED) and Depatux-M (5 mg/kg IP or 60 μg CED), and animals were processed 48 h later for H&E staining and MALDI-MSI. Ion images reflect the spatial distribution of the Cys-mcMMAF fragment used for quantitation. (**B**) Cys-mcMMAF concentrations measured in the Depatux-M CED or Depatux-M IP treated GBM6 intracranial tumors.

dosing regimen may prevent toxicity, the data presented highlight a narrow therapeutic window for Ser-T, which suggests Ser-T is unsuitable for treatment of GBM via CED infusion.

The expected EGFR-specificity for both ADCs was limited following CED infusion as compared to cell culture and systemic dosing. This presumably reflects relatively high local ADC concentrations following CED with subsequent intra- or extra-cellular toxin release. For Ser-T, linker cleavage in the brain microenvironment by hydrolytic enzymes, such as carboxylesterase 1, could result in non-specific release and subsequent cytotoxicity.[24,25] In addition, EGFR-independent internalization of intact ADCs via non-specific or Fc-γ receptor-mediated processes could lead to release of talirine or Cys-mcMMAF from normal cells.[26] While evidence for talirine-induced DNA damage in normal cells was detectable 7 days after ADC infusion, released Cys-mcMMAF was undetectable in normal brain and plasma following Depatux-M CED (Figure 6B/C) consistent with known stability of the linker/toxin chemistry.[27] Nonetheless, the incidence of peripheral neuropathy with microtubule-targeted agents raises the possibility for region-specific toxicities associated with MMAF-ADC infusions that include long white matter tracts. While

additional pre-clinical toxicity and efficacy studies will be required to understand these issues, the promising therapeutic window observed with Depatux-M in this study provides significant enthusiasm for pursuing EGFR-targeted ADC infusion by CED for this devastating disease.

Keywords

antibody-drug conjugate | convection-enhanced delivery | Depatux-M | glioblastoma | Serclutamab talirine

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References

1. Kunte S, Abraham J, Montero AJ. Novel HER2-targeted therapies for HER2-positive metastatic breast cancer. *Cancer*. 2020; 126(19):4278–4288.
2. Furnari FB, Cloughesy TF, Cavenee WK, Mischel PS. Heterogeneity of epidermal growth factor receptor signalling networks in glioblastoma. *Nat Rev Cancer*. 2015; 15(5):302–310.
3. Marin BM, Porath KA, Jain S, et al. Heterogeneous delivery across the blood-brain barrier limits the efficacy of an EGFR-targeting antibody drug conjugate in glioblastoma. *Neuro Oncol*. 2021; 23(12):2042–2053.
4. Gan HK, Kaye AH, Luwor RB. The EGFRvIII variant in glioblastoma multiforme. *J Clin Neurosci*. 2009; 16(6):748–754.
5. van den Bent M, Gan HK, Lassman AB, et al. Efficacy of depatuxizumab mafodotin (ABT-414) monotherapy in patients with EGFR-amplified, recurrent glioblastoma: results from a multi-center, international study. *Cancer Chemother Pharmacol*. 2017; 80(6):1209–1217.
6. Van Den Bent M, Eoli M, Sepulveda JM, et al. INTELLANCE 2/ EORTC 1410 randomized phase II study of Depatux-M alone and with temozolomide vs temozolomide or lomustine in recurrent EGFR amplified glioblastoma. *Neuro-Oncol*. 2020; 22(5):684–693.
7. Lassman A, Pugh S, Wang T, et al. ACTR-21. A randomized, double-blind, placebo-controlled phase 3 trial of depatuximab mafodotin (ABT-414) in epidermal growth factor receptor (EGFR) amplified (AMP) newly diagnosed glioblastoma (nGBM). *Neuro Oncol*. 2019; 21(Suppl 6):vi17.
8. Gan HK, Parakh S, Lassman AB, et al. Tumor volumes as a predictor of response to the anti-EGFR antibody drug conjugate depatuxizumab mafadotin. *Neurooncol Adv*. 2021; 3(1):vdab102.
9. Vogelbaum MA, Aghi MK. Convection-enhanced delivery for the treatment of glioblastoma. *Neuro Oncol*. 2015; 17 Suppl 2(Suppl 2):ii3–ii8.
10. Rechberger JS, Power EA, Lu VM, Zhang L, Sarkaria JN, Daniels DJ. Evaluating infusate parameters for direct drug delivery to the brainstem: a comparative study of convection-enhanced delivery versus osmotic pump delivery. *Neurosurg Focus*. 2020; 48(1):E2.
11. Gupta SK, Mladek AC, Carlson BL, et al. Discordant in vitro and in vivo chemopotentiating effects of the PARP inhibitor veliparib in temozolomide-sensitive versus -resistant glioblastoma multiforme xenografts. *Clin Cancer Res*. 2014; 20(14):3730–3741.
12. Carlson BL, Pokorny JL, Schroeder MA, Sarkaria JN. Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery. *Curr Protoc Pharmacol*. 2011; Chapter 14(14):Unit 14.16.
13. Beffinger M, Schellhammer L, Pantelyushin S, Vom Berg J. Delivery of antibodies into the murine brain via convection-enhanced delivery. *J Vis Exp*. 2019(149).
14. Eskilsson E, Røsland GV, Solecki G, et al. EGFR heterogeneity and implications for therapeutic intervention in glioblastoma. *Neuro Oncol*. 2018; 20(6):743–752.
15. Kim M, Ma DJ, Calligaris D, et al. Efficacy of the MDM2 inhibitor SAR405838 in glioblastoma is limited by poor distribution across the blood-brain barrier. *Mol Cancer Therap*. 2018; 17(9):1893–1901.
16. Li W, Yu X, Tan S, Liu W, Zhou L, Liu H. Oxymatrine inhibits non–small cell lung cancer via suppression of EGFR signaling pathway. *Cancer Med*. 2018; 7(1):208–218.
17. Westphal M, Maire CL, Lamszus K. EGFR as a target for glioblastoma treatment: an unfulfilled promise. *CNS Drugs*. 2017; 31(9):723–735.
18. Garrett TPJ, Burgess AW, Gan HK, et al. Antibodies specifically targeting a locally misfolded region of tumor associated EGFR. *Proc Natl Acad Sci*. 2009; 106(13):50825082. 5087
19. Arvanitis CD, Askoxylakis V, Guo Y, et al. Mechanisms of enhanced drug delivery in brain metastases with focused ultrasound-induced blood–tumor barrier disruption. *Proc Natl Acad Sci*. 2018; 115(37):E8717.
20. Alli S, Figueiredo CA, Golbourn B, et al. Brainstem blood brain barrier disruption using focused ultrasound: a demonstration of feasibility and enhanced doxorubicin delivery. *J Control Release*. 2018; 281:29–41.
21. Kunwar S, Chang S, Westphal M, et al. Phase III randomized trial of CED of IL13-PE38QQR vs Gliadel wafers for recurrent glioblastoma. *Neuro-Oncol*. 2010; 12(8):871–881.
22. Ung TH, Malone H, Canoll P, Bruce JN. Convection-enhanced delivery for glioblastoma: targeted delivery of antitumor therapeutics. *CNS Oncol*. 2015; 4(4):225–234.
23. Jackson PJM, Kay S, Pysz I, Thurston DE. Use of pyrrolobenzodiazepines and related covalent-binding DNA-interactive molecules as ADC payloads: is mechanism related to systemic toxicity? *Drug Discov Today Technol*. 2018; 30:71–83.

24. Ubink R, Dirksen EHC, Rouwette M, et al. Unraveling the interaction between carboxylesterase 1c and the antibody-drug conjugate SYD985: improved translational PK/PD by using Ces1c knockout mice. *Mol Cancer Therap*. 2018; 17(11):2389–2398
25. Caculitan NG, Dela Cruz Chuh J, Ma Y, et al. Cathepsin B is dispensable for cellular processing of cathepsin B-cleavable antibody-drug conjugates. *Cancer Res*. 2017; 77(24):7027–7037
26. Li F, Ulrich M, Jonas M, et al. Tumor-associated macrophages can contribute to antitumor activity through FcγR-mediated processing of antibody-drug conjugates. *Mol Cancer Therap*. 2017; 16(7):1347–1354.
27. Goss GD, Vokes EE, Gordon MS, et al. Efficacy and safety results of depatuxizumab mafodotin (ABT-414) in patients with advanced solid tumors likely to overexpress epidermal growth factor receptor. *Cancer*. 2018; 124(10):2174–2183