MerTK inhibition decreases immune suppressive glioblastoma-associated macrophages and neoangiogenesis in glioblastoma microenvironment

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Abstract

Background. Glioblastoma-associated macrophages and microglia (GAMs) are the predominant immune cells in the tumor microenvironment. Activation of MerTK, a receptor tyrosine kinase, polarizes GAMs to an immunosuppressive phenotype, promoting tumor growth. Here, the role of MerTK inhibition in the glioblastoma microenvironment is investigated in vitro and in vivo.

Methods. Effects of MRX-2843 in glioblastoma microenvironment regulation were determined in vitro by cell viability, cytokine array, in vitro tube formation, Western blotting, and wound healing assays. A syngeneic GL261 orthotopic glioblastoma mouse model was used to evaluate the survival benefit of MRX-2843 treatment. Multiplex fluorescent immunohistochemistry was used to evaluate the expression of CD206, an anti-inflammatory marker on GAMs, and angiogenesis in murine brain tumor tissues.

Results. MRX-2843 inhibited cell growth and induced apoptosis in human glioblastoma cells and decreased protein expression of phosphorylated MerTK, AKT, and ERK, which are essential for cell survival signaling. Interleukin-8 and C-C motif chemokine ligand 2, the pro-glioma and pro-angiogenic cytokines, were decreased by MRX-2843. Decreased vascular formation and numbers of immunosuppressive (CD206+) GAMs were observed following MRX-2843 treatment in vivo, suggesting that in addition to alleviating immnosuppression, MRX-2843 also inhibits neoangiogenesis in the glioma microenvironment. These results were supported by a prolonged survival in the syngeneic mouse orthotopic GL261 glioblastoma model following MRX-2843 treatment.

Conclusion. Our findings suggest that MRX-2843 has a therapeutic benefit via promoting GAM polarization away from immunosuppressive condition, inhibiting neoangiogenesis in the glioblastoma microenvironment and inducing tumor cell death.

Key Points

• The clinically available MerTK inhibitor MRX-2843 provides anti-glioma effects.
• MRX-2843 decreases immunosuppression and angiogenesis in the tumor microenvironment.
Importance of the Study

Therapeutic approaches that target the immunosuppressive tumor microenvironment, including both glioblastoma and immunosuppressive GAMs, have better chances to halt the tumor growth. Here, we investigated and demonstrated that MRX-2843, an MerTK inhibitor, induced glioblastoma cell death, polarized GAMs away from immunosuppressive state, reduced levels of IL-8 and CCL2, and decreased neoangiogenesis in the glioblastoma microenvironment. Along with blood–brain barrier penetration, the multifaceted anti-glioma effects make MRX-2843 a desirable candidate for clinical trials in glioblastoma. The findings of this study provide preclinical evidence to support the development of clinical studies of MRX-2843 in glioblastoma. Cytotoxicity was observed in a microglia and an endothelial cell line but not in human monocytes/macrophages. This underscores the need to proceed cautiously in clinical trials and look for combination therapies that induce synergistic anti-glioblastoma effects with MRX-2843 at the lowest effective dose.

Glioblastoma, the most common primary malignant brain tumor, has a poor prognosis with only a quarter of patients surviving 2 years after their diagnosis, even with the aggressive standard treatments.1 This may be partly due to the immunosuppressive glioma microenvironment which consists of several different types of cells, including glioblastoma-associated macrophages and microglia (GAMs) which are the predominant immune cells in a glioma microenvironment.2 Both microglia, the resident macrophages and all other macrophages in the central nervous system (CNS) play pivotal roles in gliomagenesis and neovascularogenesis, a hallmark of glioblastoma.3 Therefore, promising therapies targeting multiple cell types in the tumor microenvironment may provide durable responses to control or eliminate glioblastoma.

Receptor tyrosine kinase MerTK is highly expressed in GAMs and it belongs to the TAM family including TYRO3 and AXL. MerTK is activated by ligands such as protein S (PROS1), galectin-3, and growth arrest-specific 6 (GAS6), which stimulates phagocytosis and efferocytosis.4,5 Activation of MerTK in macrophages is known to stimulate repair of tissue damage and maintain homeostasis in physiological conditions. Activated MerTK signaling contributes to the immunosuppressive glioma microenvironment and promotes tumor progression through shifting microglia and macrophages to an immune-paralyzing and tumor-promoting phenotype.6-9 We and others have shown that GAMs are more infiltrating in recurrent glioblastoma and display immunosuppressive phenotypes, suggesting that MerTK inhibition may alleviate immune suppression in the tumor microenvironment of aggressive glioblastomas.7,10

Recent studies showed that increasing cytokine secretion such as Interleukin-8 (IL-8) and C-C motif chemokine ligand 2 (CCL2) within the tumor microenvironment promotes gliomagenesis.11,12 IL-8, also known as CXCL8, is a pro-inflammatory CXC chemokine that activates multiple intracellular signaling pathways by binding to G protein-coupled receptors (CXCR1 and CXCR2). Increased expression of IL-8 in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages within the tumor microenvironment is associated with tumor progression and promotion of neovascular formation.13-15 Therefore, inhibiting the effects of IL-8 signaling may be a significant therapeutic intervention in targeting the glioma microenvironment.13 CCL2, also called monocyte chemoattractant protein-1, is one of the C-C chemokines subfamily, and CCL2–CCR2 axis signaling is involved in modulating migration and penetrative infiltration of monocytes/macrophages from the blood stream.16 Secreted CCL2 from tumor cells and a variety of immune cells such as macrophages can trigger angiogenesis and tumor cell proliferation, and CCL2 production by immunosuppressive GAMs is associated with poor clinical outcome.17,18 Low expression of CCL2 in glioblastoma was shown to be associated with increased overall survival compared to patients with high expression of CCL2.19

Previously, our group demonstrated that MerTK receptor inhibitor UNC2025 was able to reduce the immunosuppressive macrophages in mouse brain tumor tissue and cause modest cytotoxicity in tumor cells, suggesting the therapeutic potential of MerTK inhibition for brain tumors through targeting both tumor cells and tumor microenvironment. MRX-2843 is a structurally related MerTK inhibitor that is progressing through phase I clinical trials to establish tolerability and pharmacokinetics in patients with late-stage solid tumors, other than brain tumors. In order to understand the impact of MRX-2843 on glioblastoma and the tumor microenvironment, we carried out a series of preclinical studies to investigate whether MRX-2843 has anti-glioma effects through the direct cytotoxicity on glioma cells and the tumor microenvironment modulation, focusing on the GAM polarization toward a pro-inflammatory phenotype, altering cytokine production, and inhibiting neoangiogenesis.

Materials and Methods

Reagents, Antibodies, Cell Lines, and Culture Conditions

A list of reagents, antibodies, and cell lines with their culture conditions are described in detail in Supplementary Materials and Methods.
Differentiation and Stimulation of Macrophages

Adult human elutriated monocytes from healthy donors were obtained by an Institutional Review Board approved protocol from the National Institutes of Health Blood Bank (Bethesda, MD). Human monocytes were cultured and differentiated by adding granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor. Macrophages were then stimulated into pro-inflammatory and anti-inflammatory macrophages using interferon-gamma and interleukin-4, respectively. Additional protocol details are described in Supplementary Materials and Methods.

Immunoblot Analysis

The cells including GSC923, GSC407, U251, GL261, macrophages, and human brain microvascular endothelial cell (HBMEC) were treated with 100 nM of MRX-2843 prior to the cell harvest. The cell lysates were processed with 1× sample buffer and Western blotting was performed as described previously.19

Cell Viability Assay

The cell viability was determined using a cell counting method. All cell lines were treated with MRX-2843 for 48 h prior to cell counting using a Beckman Coulter Vi-CELL XR cell viability analyzer.

Clonogenic Assay

The clonogenic assay was performed using U251, GSC923, and GSC407 as described in Supplementary Materials and Methods. Briefly, cells were treated with MRX-2843 for 48 h before incubation in fresh medium for 7–14 days before colonies were counted for analysis of survival fractions.

Cytokine Analysis

Cytokine profile was analyzed using the Proteome Profiler Array (Human Cytokine Array Panel A, ARY005B) from R&D Systems per manufacturer’s instructions. Cytokine and chemokine protein levels were measured from culture media collected from cells with or without drug treatments.

Flow Cytometry Analysis

Immunofluorescence staining and fluorescence activated cell sorting analyses were performed as previously described.20 Macrophages were collected after 48 h treatment and processed as described in Supplementary Materials and Methods. The data were acquired on a BD LSRFortessa SORP analyzer and results were analyzed using FlowJo software (Becton, Dickinson & Company).

Wound Healing Migration Assays

The wound healing assay was performed as described previously.21 In brief, the macrophages, HMC-3 and U251, were seeded, treated with 100 nM of MRX-2843, and imaged as described in Supplementary Materials and Methods. Percentage wound closure was measured after 24 h drug treatment.

In Vitro Tube Formation Assays

The Cultrex In Vitro Angiogenesis Assay Tube Formation Kit from Trevigen (3470-096-K) was used to perform two-dimensional endothelial cell tube formation according to the manufacturer protocol, as described in Supplementary Materials and Methods.

Orthotopic Glioblastoma Mouse Model

A syngeneic orthotopic glioblastoma mouse model was used to evaluate the treatment effects of MRX-2843 in vivo according to an approved animal study proposal by NCI-Animal Use and Care Committee and as described previously.22 MRX-2843 administration started on day 7 (oral gavage daily, 50 mg/kg) after intracranial injection of GL261 cells. When the animals reached end points, they were euthanized by perfusion with 4% PFA under anesthesia, and the brains were dissected for histopathologic examination and immunohistochemistry (IHC) analysis.

Multiplex Fluorescence IHC

Multiplex fluorescence IHC was performed on 10-μm-thick formalin-fixed cryosections sourced from mouse brain tissues implanted with mCherry-expressed GL261 glioblastoma using up to 5 iterative rounds of sequential immunostaining with select antibody panels tailored to phenotype relevant neuroinflammatory, glioblastoma-associated, and vascular cell types. Detailed procedure is described in Supplementary Materials and Methods.

Statistical Analysis

Statistical analyses were performed using a GraphPad Prism software (Version 6.05, GraphPad Software, Inc.) and considered significant at *P < .05, **P < .01, ***P < .001, or ****P < .0001 level. Data are shown as mean ± SEM.
One-way ANOVA or independent Student’s t-test was used for statistical comparisons.

Results

MRX-2843 Induces Apoptosis in Human Glioblastoma Cells

Previously, we showed that UNC2025 inhibited cell growth and induced cell death in glioblastoma. Similar to UNC2025, we found that MRX-2843 also crosses the blood–brain barrier (BBB) (Supplementary Figure S1). Glioblastoma cell line U251 and primary patient-derived glioma stem cell (GSC) lines GSC923 and GSC407 were used to evaluate the cytotoxicity of MRX-2843 by determining the cell viability. We showed that the half maximal effective concentration (EC50) values in U251, GSC923, and GSC407 were 95.5, 288.1, and 217.7 nM, respectively, after 48 h treatment with MRX-2843 (Figure 1A). A decrease of colony formation was observed 7–14 days after U251, GSC923, and GSC407 cells were treated with MRX-2843 at both 100 or 500 nM for only 48 h (Figure 1B), suggesting that MRX-2843 suppresses long-term glioma cell viability after short-term drug exposure. In order to validate the target effects of MRX-2843 on MerTK and its downstream signaling pathways, the glioma cells were treated with 100 nM of MRX-2843 for 4 h. Even when the cells were exposed to this low dose of MRX-2843, the phosphorylated MerTK was downregulated by 27% in U251, 46% in GSC923, and 36% in GSC407, when expression of phosphorylated MerTK was normalized to the total MerTK expression (Figure 1C). Phosphorylation of both AKT and ERK was found to be decreased in MRX-2843-treated cells at 24 h (Figure 1C). The apoptotic signaling including cleaved-PARP, cleaved-caspase-3, and phosphorylation of γ-H2AX was found to be increased in MRX-2843-treated U251, GSC923, and GSC407 cells at 48 h, suggesting that MRX-2843 induces apoptosis in glioblastoma cells (Figure 1D).

The Anti-glioma Effects of MRX-2843 in Mouse Glioma In Vitro and In Vivo

A GL261 syngeneic mouse glioblastoma model was used to evaluate the survival benefit of MRX-2843 treatment in vivo. First, we examined the sensitivity of GL261 to MRX-2843 in cell-based assays and found the EC50 of MRX-2843 for growth inhibition in GL261 was 88.6 nM, suggesting that GL261 is sensitive to MRX-2843 treatment compared to the human GSC lines that were assessed in this study (Figure 2A, panel a). When normalized to their total protein expression, the expression of phosphorylated-MerTK, phosphor-AKT, and phosphor-ERK was found to be decreased by 21%, 15%, and 15%, respectively, in the MRX-2843-treated GL261 cells (Figure 2A, panel b), suggesting that MRX-2843 inhibited GL261 cell growth and decreased the activity of MerTK signaling. Next, we examined whether MRX-2843 has anti-glioma effects in the orthotopic syngeneic mouse glioblastoma model with mCherry GL261 by analyzing the survival in mice with or without MRX-2843 treatment. The median survival was 26 days and 23 days in MRX-2843-treated and vehicle-treated groups, respectively, suggesting a modest survival benefit of MRX-2843 in mouse glioblastoma model (Figure 2B). In addition to direct tumor growth inhibition, several other mechanisms may contribute to survival benefit in this model such as decreasing angiogenesis, increasing immune cell infiltration, and creating a less immunosuppressive tumor microenvironment. Here, we examined the effect of MRX-2843 on neoangiogenesis in vivo. Vasculature was stained using a group of markers including SMA, CD31, and collagen IV. Brain tumor area was defined by mCherry expressed by the tumor cells. Decreased vessel area in the brain tumor was observed after mice were treated with MRX-2843 for 14 days (n = 2) when compared with control groups, suggesting that MRX-2843 decreases neoangiogenesis in vivo, measured by the decreased vessel area in the brain tumor tissues in the GL261 mouse model (Figure 2C and Supplementary Figure S2A).

Previously, we showed that UNC2025 treatment combined with radiation decreased the expression of CD206 in macrophages. Given the modest but statistically significant survival benefit of MRX-2843 as single-agent treatment, we tested whether MRX-2843 treatment modulated the macrophage phenotype within the glioma microenvironment by multiplex IHC. We found that the CD206 marker could identify both macrophage and TMEM119-positive microglia cell populations (Supplementary Figure S2B). We used CD11c to further distinguish macrophages and activated microglia from the resting microglia. The density of CD206-positive cells was analyzed as described in Materials and Methods (see the Multiplex Fluorescence IHC Image Analysis section). In the treatment group, CD206 expression was decreased within the tumor site compared to control, and the density of CD206-positive microglia was increased in the surrounding tissue. These data indicate that MRX-2843 decreases immunosuppressive macrophages and microglia (CD206+) in the tumor microenvironment in vivo (Figure 2D).

MRX-2843 Decreased the Anti-inflammatory Macrophages in Glioma Microenvironment

Since the decrease of anti-inflammatory macrophages was observed in vivo, we next investigated whether MRX-2843 is capable of reversing the immunosuppressive phenotype of macrophages into pro-inflammatory phenotype within the tumor microenvironment. We set up a cell-based model (Figure 3A, panel a) where we differentiated and stimulated human monocytes from the healthy donors to become either anti-inflammatory or pro-inflammatory macrophages, defined by CD163high/CD206high or CD80high/CD86high, respectively (Supplementary Figure S3), and then treated macrophages with serially diluted MRX-2843 for 48 h. We found that MRX-2843 does not induce cell death in either pro- or anti-inflammatory macrophages as determined by cell counting methods (Figure 3A). We further examined if MRX-2843 modulates the macrophage polarization in the glioma microenvironment, mimicked by adding U251-conditioned medium. MRX-2843 decreased CD163high/CD206high positive anti-inflammatory macrophages and increased CD80high/CD86high positive pro-inflammatory macrophages.
macrophages. Furthermore, we found that MRX-2843 decreased the anti-inflammatory macrophages in the gliomamimic microenvironment, but there were no changes of pro-inflammatory macrophages (Figure 3B and C). Increasing protein expression of phosphorylated Stat3, Stat6, and Arginase I is suggestive of GAM polarization toward an anti-inflammatory phenotype.5,24,25 Consistent with our previous findings of decreased anti-inflammatory macrophages, we found decreased MerTK signaling and decreased phospho-Stat3, phospho-Stat6, and Arginase I expression in MRX-2843-treated anti-inflammatory macrophages (Figure 3D). Decreased Stat3 activity was also demonstrated in pro-inflammatory macrophages (Figure 3E). These data suggest that inhibiting MerTK by MRX-2843 in macrophages alleviates their immune-paralyzing phenotype which makes the tumor microenvironment less immunosuppressive.

MRX-2843 Decreased Cytokine IL-8 in Glioma Tumor Microenvironment

Cytokines secreted from immune cells and tumors that promote gliomagenesis are the key to creating an immunosuppressive tumor microenvironment.13 Cytokine profiling of the macrophages was performed using cytokine arrays. Decreased secretion of IL-8 and CCL2 was found in the supernatant obtained from MRX-2843-treated anti-inflammatory and pro-inflammatory macrophages (Figure 4A and B, panel a). Consistently, we also observed decreased intracellular cytokine production of IL-8 in MRX-2843-treated anti-inflammatory macrophages and pro-inflammatory macrophages (Figure 4A and B, panel b). Although a decrease in CCL2 was noted in supernatant from drug-treated anti-inflammatory macrophages (Figure 4A, panel a), the intracellular protein expression of CCL2 was undetectable in either anti- or pro-inflammatory macrophages by Western blot. Furthermore, we examined the cytokine secretion of MRX-2843-treated U251 cells and found that MRX-2843 decreased secretion of IL-8 and CCL2 at 100 nM (Figure 4C). MRX-2843 inhibits intracellular cytokine production of IL-8 in U251, GSC923, and GSC407 cells and CCL2 in U251 and GSC923 but not in GSC407 cells (Figure 4D and Supplementary Figure S4). We demonstrate that MRX-2843 decreases cytokine secretion of IL-8 within the in vitro tumor microenvironment, which may lead to a less immunosuppressive microenvironment.

MRX-2843 Decreases Immunosuppressive Phenotype of Microgla and Induces Microgla Cell Migration

Resident microglia and macrophages are the predominant immune cells in a glioma tumor microenvironment. We examined the cell viability in a human microglial cell line HMC-3, which was established through SV-40-dependent immortalization of human microglia cells.26 We found that the cell viability of HMC-3 was decreased 40% compared to control following treatment with 100 nM MRX-2843, indicating that MRX-2843 has a cytotoxic effect in HMC-3 cells (Figure 5A). Cytokine profiling of HMC-3 was conducted, which revealed that MRX-2843 caused a reduced secretion of IL-8 in HMC-3 (Figure 5B). We have observed that the density of CD206-positive microglia was increased in the surrounding tissue in vivo (Figure 2D), suggesting migration of microglia to the peri-tumoral region. As the resident immune cells of the CNS, microgla cells consistently surveil the microenvironment and become activated in various CNS diseases.26 To determine if migration of the immune cells is relevant to the brain tumor microenvironment, we further investigated whether MRX-2843 affects the cell migration of microglia, macrophages, and U251 using the in vitro wound healing migration assay. Regardless of adding U251 conditioned medium, MRX-2843 increases cell migration in HMC-3 cells (Figure 5C, panels a and b), but neither in anti-inflammatory macrophage (Figure 5C, panels c and d) nor U251 cells (Figure 5C, panels e and f). Interestingly, pro-inflammatory macrophages did not have the ability to migrate as determined by wound healing assays (Supplementary Figure S5). These data suggest that MRX-2843 also affects the resident microglia in addition to GAM.

MRX-2843 Inhibits Angiogenesis in Gliobastoma Microenvironment

We found that MRX-2843 inhibits neoangiogenesis in the GL261 mouse glioblastoma model in vivo. To determine whether MRX-2843 has an impact on cell growth of HBMECs, the cells were treated with different dosages of MRX-2843. The sensitivity of HBMEC to MRX-2843 was found to be similar to HMC-3 cells (Figure 6A). Next, we tested if MRX-2843 affects vessel tube formation by performing two-dimensional in vitro tube formation assays in 3 different conditions including endothelium cell medium, 30% DMEM, and 30% of conditioned medium. Decreased branch-point counting was found in MRX-2843-treated HBMECs after 4 and 24 h, suggesting that MRX-2843 inhibits vascular formation (Figure 6B). MerTK activity and downstream AKT signaling were also decreased in HBMECs (Figure 6C) following MRX-2843 treatment, while HBMEC migration was not affected by MRX-2843 (Supplementary Figure S6), indicating that AKT downregulation in MRX-2843-treated HBMECs results in decreased angiogenesis but not cell migration. We demonstrated that MRX-2843 inhibits gliomagenesis partly through decreasing neoangiogenesis.

Discussion

Drug discovery for brain tumor treatments has been extensively conducted; however, little progress has been made. Challenges for finding therapies to control or eradicate glioblastoma exist in part due to the unique intrinsic tumor cell resistance in addition to the impact of the tumor microenvironment, including the anti-inflammatory immune network and neovascular formation. Thus, the immune and vascular components of the tumor microenvironment are important factors to focus on in therapy development.27,28 GAMs, as the key players in the glioblastoma
Figure 1. MRX-2843 induces human glioblastoma cell death. (A) The cytotoxicity of MRX-2843 in glioblastoma cells. The dose–response curves and the EC50 values of MRX-2843 in U251, GSC923, and GSC407 cells are shown in panels a and b, respectively. (B) MRX-2843 decreases the colony formation in U251, GSC923, and GSC407 cells (panels a, c, and e) after treatment with 100 and 500 nM of MRX-2843 for 48 h. Survival fractions were derived from the numbers of colony formation 7-14 days after treatments relative to control (panels b, d, and f). Data from 3 independent biological replicates were shown, each including 4 technical replicates. (C) MRX-2843 inhibits protein expression of MerTK signaling in U251, GSC923, and GSC407 cells following treatment with 100 nM of MRX-2843 for 4 and 24 h (panels a–f). (D) MRX-2843 induces glioblastoma apoptosis. The protein expressions of cleaved-PARP, cleaved caspase-3, and phosphorylated H2AX (Ser139) were detected by Western blot after MRX-2843 treatment for 48 h.
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Figure 2. The anti-glioma effects of MRX-2843 in mouse glioma in vitro and in vivo. (A) MRX-2843 inhibits GL261 cell growth. a, The dose–response curve of MRX-2843 in GL261. b, MRX-2843 down-regulates MerTK signaling in GL261 cells. (B) MRX-2843 treatment at 50 mg/kg P.O. daily significantly increased the overall survival in a syngeneic orthotopic mouse glioblastoma model implanted with mCherry-GL261 cells. a, A schematic illustration of drug administration and procedure created with BioRender.com. b, The median survival in the vehicle-treated group is 23 days while the MRX-2843-treated group is 26 days. The result was analyzed using Log-rank test for trends in GraphPad Prism software (Chi square = 5.281, df=1, *P = .0216). (C) MRX-2843 decreases neoangiogenesis within the tumor microenvironment in vivo. Multiplex IHC staining of GL261 tumors (mCherry expression, red) and a vasculature panel, including CD31 (an endothelial cell marker, green), collagen IV, CD11c / CD206 were used to evaluate the vasculature in the whole brain (panel a) and tumor tissues (panels b and c). The statistical data of vessel area were shown in panel d as described in the Multiplex Fluorescence IHC image analysis section of Materials and Methods. (D) MRX-2843 decreases immunosuppressive macrophages (CD206+) in tumor microenvironment. CD11c/CD206-positive macrophages in the whole brain (panel a) and tumor tissues (panels b and c) were observed and CD206 fluorescent intensity was analyzed quantitatively using image J software (panel d). CD206+ cells were shown as green and CD11c+ cells were shown as purple. The scale bar represents 1 mm (C and D, panel a) and 300 µm (C and D, panels b and c).
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We demonstrated that MRX-2843 has direct effects on all important players in the glioblastoma microenvironment with the goal of understanding the full spectrum of the effects of MRX-2843. We designed to achieve a higher drug concentration in brain tumor tissue versus plasma (Supplementary Figure S1), was investigated to achieve a higher drug concentration in brain tumor tissue.

MRX-2843, a clinical-stage MerTK inhibitor which can be differentiated and stimulated into anti- or pro-inflammatory macrophages and the process was illustrated in panel a and created with BioRender.com. The anti-inflammatory (panel b) and pro-inflammatory (panel c) macrophages were treated with indicated dosages of MRX-2843. The expression of cell markers CD206 and CD163 on anti-inflammatory macrophages (CD163/CD206 double positive cell population) is significantly decreased by MRX-2843 treatment when co-cultured with human glioblastoma conditioned medium (n = 15). The flow cytometry analysis was carried out to determine the intensity of cell markers. Fold changes were calculated by comparing the median fluorescence intensity (MFI) of the MRX-2843-treated groups to the MFI of their respective control groups. We set the fold change of each individual control group to 1. (D and E) Western blotting analysis showing the down-regulated MerTK signaling in MRX-2843-treated anti-inflammatory (D) and pro-inflammatory (E) macrophages.

MRX-2843 Targets Both Glioblastoma Cells and GAMs

Our previous study of UNC2025, a nonclinical MerTK receptor inhibitor, suggested the therapeutic potential of MerTK inhibition for brain tumors through targeting both tumor cells and the tumor microenvironment. In this current study, MRX-2843, a clinical-stage MerTK inhibitor which can achieve a higher drug concentration in brain tumor tissue versus plasma (Supplementary Figure S1), was investigated with the goal of understanding the full spectrum of the effects on all important players in the glioblastoma microenvironment. We demonstrated that MRX-2843 has direct anti-glioma effects, inhibiting tumor cell proliferation and causing cell death. In addition, we demonstrated that MRX-2843 decreased the number of anti-inflammatory (CD163high/CD206high) macrophages and increased pro-inflammatory (CD80high/CD86high) macrophages using differentiated and stimulated human monocytes from healthy donors, suggesting that MRX-2843 induces a less immunosuppressive microenvironment. In an orthotopic syngeneic mouse glioblastoma model, MRX-2843 treatment extended animal survival and decreased vascular formation and CD206/CD11c-positive cells within the tumor microenvironment compared to the untreated mice.

After infiltration into the tumor microenvironment, GAMs become polarized toward an immunosuppressive, anti-glioma phenotype, while killing tumor cells, is likely required to prevent tumor progression. Reeducating GAMs to adopt a less immunosuppressive phenotype in the tumor microenvironment, while killing tumor cells, is likely required to prevent tumor progression.

Microenvironment, can be educated by glioblastoma cells to produce an immunosuppressive environment.2,29 Hence, modulation and reeducation of GAMs in the glioblastoma microenvironment are considered a promising route to therapy.

Figure 3. MRX-2843 decreases the anti-inflammatory macrophages in glioma microenvironment. (A) The monocytes from healthy donors were differentiated and stimulated into anti- or pro-inflammatory macrophages and the process was illustrated in panel a and created with BioRender.com.
antitumor strategy. Both Stat3 and Stat6 signaling are known to promote tumor progression and drive the immunosuppressive polarization of macrophages.\cite{36-38} We found that MRX-2843 decreased the production of CCL2 and IL-8 in both GAMs and glioblastoma cells. In addition, we demonstrated that the activity of Stat3 and Stat6 was decreased in the anti-inflammatory macrophages after MRX-2843 treatment, suggesting that MerTK inhibition reverses the immunosuppressive tumor microenvironment in glioblastoma.

**MRX-2843 Has the Potential to Suppress Tumor Vascular Formation**

Tumor-associated macrophages recruit pro-angiogenic factors and promote angiogenesis in tumors.\cite{39} In our study, multiplex IHC of the mouse brain tumor samples demonstrated decreased vascular formation in MRX-2843-treated mice. A 2D in vitro tube formation assay using brain microvascular endothelial cells also showed that MRX-2843 decreased vascular formation, suggesting the anti-angiogenic effect of MerTK inhibition (Figures 2 and 6). Increasing evidence supports that cytokine secretion of IL-8 promotes neoangiogenesis in vitro and in vivo,\cite{2} and high expression of many potently anti-inflammatory and immunosuppressive factors such as IL-8 in glioblastoma patients has been found to be associated with dismal survival.\cite{1} Both secreted and intracellular levels of IL-8 were found to be decreased in macrophage, microglial and tumor cells, consistent with the anti-angiogenic effect of MRX-2843 in glioblastoma (Figures 4 and 5). Our findings suggest that MRX-2843 has an anti-glioma effect in part through inhibiting vascular formation.

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**Figure 4.** MRX-2843 decreased cytokine secretion of IL-8 in glioma tumor microenvironment. (A) Decreased cytokine secretion of IL-8 (green) and CCL2 (purple) and intracellular cytokine production of IL-8 in MRX-2843-treated anti-inflammatory macrophages for 48 h followed by cytokine array assay of culture medium (panel a) and Western blot (panel b), respectively. The numbers below the bands represent the normalized band intensity of IL-8 to beta-actin of each Western blot in control and treatment groups. (B) Cytokine array assays (panel a) and Western blotting (panel b) were used to determine cytokine secretion and intracellular cytokine production in MRX-2843-treated pro-inflammatory macrophages. (C) MRX-2843 decreases cytokine secretion of IL-8 and CCL2 in culture medium of U251 cells. (D) MRX-2843 inhibits intracellular cytokine production in U251 cells after treatment for 4 h.
Considerations in Developing MRX-2843 as a Therapeutic Approach in Glioblastoma

BBB penetration is an obstacle for drug development for CNS tumors. Here, we showed that MRX-2843 is capable of BBB penetration with an accumulated concentration of MRX-2843 in brain tumor tissue, which is about 5 times that of the plasma level (Supplementary Figure S1), suggesting a better pharmacokinetic feature compared to UNC2025.
Unexpectedly, we found cytotoxicity of MRX-2843 in the microglia and the endothelial cell line at the in vitro active doses. This has not been the case with other myeloid or endothelial cells tested under other conditions (H. S. Earp, personal communication). This may represent an addiction of these cell lines to MerTK signaling or an off-target effect to which these cell lines are sensitive. However, it does raise the question of whether combination therapy with other agents would demonstrate anti-glioma effects of MRX-2843 at a lower dose. Potential drug candidates for combination with MRX-2843 include therapies that target GAMs and macrophage-mediated angiogenesis and enhance the communication between innate and adaptive immunity. Currently, there is an ongoing phase I trial investigating MRX-2843 in adults with advanced and/or metastatic solid tumors (NCT03510104), which may provide insight into the potential of MRX-2843 as a therapeutic strategy in glioblastomas.

In summary, our findings suggest that MRX-2843 modulates the glioblastoma microenvironment through inducing tumor cell apoptosis, decreasing immuno-suppressive GAMs, suppressing pro-glioma cytokines, decreasing neoangiogenesis, and may have a therapeutic benefit, particularly in combination with therapies that have synergistic anti-cancer effects without enhancing toxicities.

**Supplementary Data**

Supplementary data are available at *Neuro-Oncology Advances* online.

**Keywords**

glioblastoma | immune modulation | MerTK | MRX-2843 | neoangiogenesis
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Conflict of interest statement. MRX-2843 was supplied by the Wang and Frye UNC academic laboratories. H.S.E. and S.V.F. are founders, stockholders, and Board members of the UNC start-up Meryx Inc, which is commercializing MerTK UNC intellectual property and conducting a phase I clinical trial of MerTK tyrosine kinase inhibitors. X.W. also holds stock in Meryx Inc.

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