The Novel Phospholipid Ether Analog CLR1404 Decreases Glioblastoma Stem Cell Proliferation, Suppresses GBM Growth, and Improves Survival

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Abstract

Introduction: Most patients treated for glioblastoma multiforme (GBM), the most common primary brain malignancy, have less than 2 years survival due to rapid recurrence. The GBM stem cell (GSC) sub-population exhibits therapeutic resistance and is hypothesized to drive tumor recurrence. Therefore, GSC-specific targeting is likely critical for improving outcome. The phospholipid ether CLR1404 and its analogs exhibit cancer cell-specific uptake and prolonged retention in 57/61 cancer cell lines and xenografts (including GBM) due to affinity for cancer cell lipid rafts. In this study, we investigated the therapeutic potential of CLR1404 against GBM and its GSC subpopulation.

Methods: Multiple sphere-forming GSC lines were isolated from patient specimens with IRB approval, and rigorously validated for self-renewal, multi-lineage potential, and high efficiency orthotopic tumor initiation in NOD-SCID mice. CLR1404 and analogs were obtained from Novelos Therapeutics (Madison, WI). Proliferation assays were performed by addition of CLR1404 analogs in serum-free medium for 24 hours. To test effects on stem cell properties, GSCs were treated with CLR1404, dissociated to single cells, plated at 500-1000 cells in a 96-well plate, and allowed to form spheres (≈2-4 weeks). CLR1404 inhibition of the AKT oncogenic signaling pathway in GSCs was assayed with immunoblot analysis. In vivo, GSCs were pre-treated for 24 hours with CLR1404 analogs prior to orthotopic injection of 200,000 live cells into immunodeficient mice. Subsequent survival curves were then constructed.

Results and Conclusion: CLR1404 anti-proliferative effects were seen on all 7 different GSC and GBM lines tested with IC_{50} values ranging between 5-10 μM using MTS assay; control normal differentiated neural cells exhibited an IC_{50} of approximately 40 μM. CLR1404 treatment also decreased sphere-forming ability of multiple GSC lines with IC_{50} values between 5-10 μM. CLR1404 inhibition of AKT activation was observed using immunoblot analysis. CLR1404 pre-treatment of GSCs significantly increased survival time in an orthotopic mouse model (Control: 59±6.1 days; CLR1404: 94±4.4 days), suggesting CLR1404 treatment of the GSC tumor initiating cells improves outcome. This data of CLR1404's therapeutic potential against GBM and its GSCs, combined with previously demonstrated tumor cell targeting specificity of CLR1404 and its analogs, provides strong evidence for the potential of novel CLR1404-based therapies to improve GBM outcomes.

Introduction

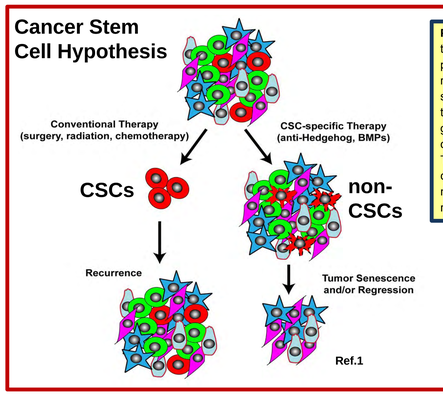


Figure 1. Cancer stem cell (CSC) hypothesis. Patients treated for glioblastoma multiforme (GBM), the most common primary brain malignancy, have less than 2 year survival due to rapid recurrence. Multiple studies have now reported a small sub-population of cancer stem cells (CSCs) within brain tumors that are highly efficient at initiating tumors and drive tumor growth. This sub-population has also been reported resistant to common treatments such as chemotherapy and radiation. Therefore, newly emerging therapies must consider this cancer cell sub-population. Ideal therapies will affect both CSC and non-CSC populations for maximal and lasting anti-tumor response.

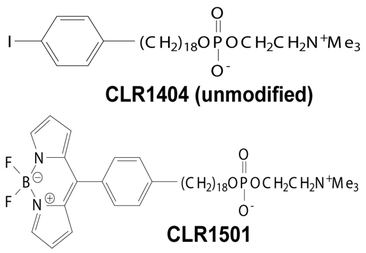
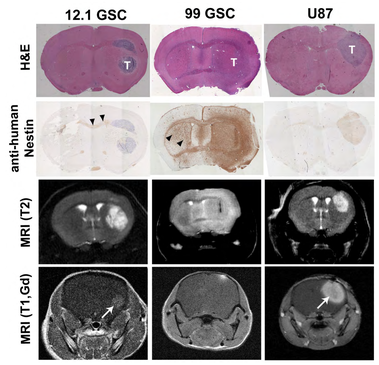


Figure 2. Structures of novel CLR1404 and analogs. Multiple phospholipid ether analogs were optimized for tumor cell uptake and retention, resulting in the lead candidate CLR1404 (Ref 2). CLR1404 exhibits selective uptake and retention in 57/61 cancer cell lines as well as xenograft and spontaneous mouse cancer models. In the fluorescent analog CLR1501, a green fluorescent BODIPY moiety is attached and has demonstrated equivalent tumor selectivity and retention to the parent compound.

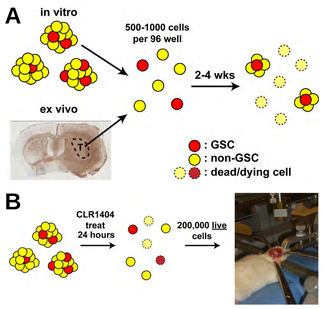
Efficacy testing of CLR1404 and analogs against glioblastoma cancer stem cells (GSCs)

I. Isolation and validation of GSCs



- **Glioblastoma cancer stem cells (GSCs)** were isolated via sphere formation from patient specimens with IRB approval from University of Wisconsin - Madison
- Validated by sphere-formation, multipotency to multiple neural lineages, and highly efficient GBM formation in immunodeficient mice
- GSC- derived tumors (T) (line 12.1 and 99 GSC) exhibit enhanced invasion into surrounding normal brain (arrowheads), a major hallmark of human GBM, evidenced by human specific nestin staining (brown). These invasive GSC-derived GBM are in sharp contrast to well delineated U87 cell line xenografts.

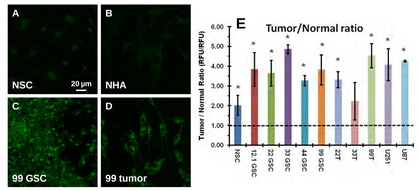
II. Therapeutic efficacy of CLR1404 analogs against GSCs and GBM



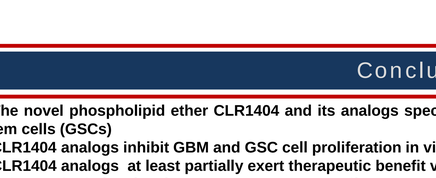
- **CLR1501 imaging:** Confocal microscopy was performed on Nikon A1R with proper filter sets. In vitro, GSCs and GBM cells were plated on coverslips, incubated overnight with 5 μM CLR1501, rinsed extensively, fixed with 4% paraformaldehyde, and imaged. In vivo, brains were perfuse fixed and frozen sectioned at 20 μM. Standard immunohistochemical methods were used to label CD133 (Abcam #ab19898). For flow cytometry, GSC spheres were labeled overnight with 5 μM CLR1501, dissociated, co-labeled with anti-AC/CD133 antibody (Miltenyi Biotec), and analyzed on a FACSCalibur (Becton Dickinson), with proper gating using isotype controls. Normal neural stem cells (NSCs) and human astrocytes (NHA) were used as cellular controls.
- **Proliferation assay**: GSC spheres or GBM cell lines were dissociated and plated at optimal density (5k-50k cells) in 96 well plates coated with laminin. After overnight attachment, cells were rinsed and treated with CLR1404 or vehicle (in medium *minus* bovine serum albumin) for 24 hrs. MTS assay was performed with proper controls, and data presented as normalized to vehicle.
- **Sphere-forming assay (A)**: GSC spheres (in vitro) or xenograft GBM (ex vivo) were enzymatically dissociated. Cells were plated at optimal density (500-1000 cells) into a 96 well plate. Upon formation of 50 μm diameter spheres (≈ 2-4 wks), GSC spheres were manually counted.
- **Survival analyses (B)**: GSC spheres were pre-treated with 60 μM of CLR1404 analog or vehicle for 24 hrs prior to orthotopic injection of 200,000 live cells into immunodeficient mice. Upon neurological symptoms in mice, the tumors were visualized using magnetic resonance imaging (MRI, T2-weighted with no contrast). Mice were subsequently followed until moribund and survival curves constructed.

CLR1404 analogs selectively label GBM and GSCs

I. CLR1404 analogs differentially label GSCs and GBM cells in vitro



II. CLR1404 analogs label both CD133⁺ and CD133⁻ GSCs



III. CLR1404 analogs label and are retained long-term by GSCs in vivo

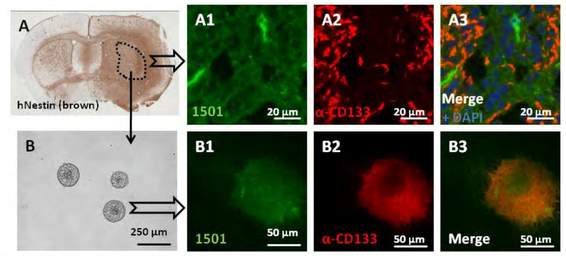
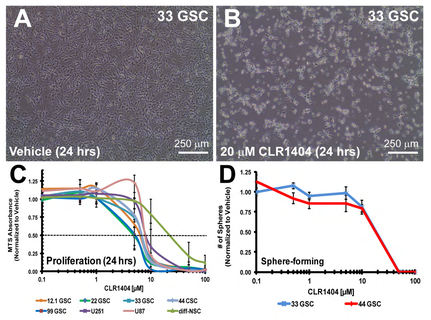


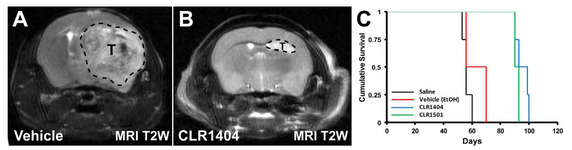
Figure 3. (I). Elevated CLR1501 (green) fluorescence was observed in GSCs (C) and GBM cells (D) compared to control neural stem cells (NSCs) (A) and human astrocytes (NHA) (B) by confocal microscopy after 24 hr incubation with 5 μM. Flow cytometry demonstrated significantly greater CLR1501 labeling in 9 of 10 GSC and GBM cell lines compared to NHAs (dotted line) (E; *: p<0.05). **(II).** After 24 hour incubation with CLR1501, the CD133⁺ and CD133⁻ GSC sub-populations were analyzed by flow cytometry. Extensive CLR1501 labeling was demonstrated for both CD133⁺ and CD133⁻ GSC populations (A). In 4 of 5 tested lines, CD133⁺ GSCs exhibited significantly higher labeling than CD133⁻ counterparts (B; *:p<0.05). **(III)** CLR1501 (1 mg) was injected 24 hrs prior to sacrifice of mice bearing GSC-derived xenografts (A: human-specific nestin immunolabeling of engrafted cells). Half the tumor analyzed by confocal microscopy demonstrated extensive CLR1501 labeling of both CD133⁺ and CD133⁻ GBM cells (A1-A3). The other tumor half was enzymatically dissociated into sphere culture, and GSC spheres developed in 2-3 weeks (B). These GSC spheres retained CLR1501 labeling as evidenced with confocal microscopy, which again co-labeled with CD133⁺ cells (B1-B3). Taken together, these results provide strong evidence for successful GSC labeling in vitro and in vivo, with CLR1501 retained long-term (weeks) and distributed to GSC progeny.

CLR1404 analogs exert therapeutic benefit against GBM and GSCs

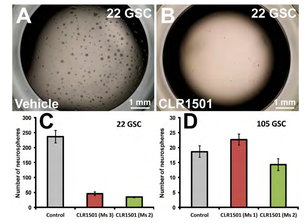
I. CLR1404 analogs inhibit proliferation and sphere formation in vitro



II. CLR1404 analogs suppress GBM growth and improve survival



III. In vivo CLR1404 analog treatment inhibits GSC sphere formation ex vivo



IV. CLR1404 analogs inhibit AKT activation

Figure 4. (I) 7 GSC and GBM cell lines were treated with CLR1404 to construct dose response curves. After 24 hr incubation, fewer remaining cells were observed for all tested lines (B) compared to vehicle controls (A). Proliferation was quantified using MTS assay, and demonstrated IC_{50} values from approximately 5-10 μM against GSC and GBM lines (C), while normal differentiated neural cells exhibited an IC_{50} of approximately 40 μM. CLR1404 treatment inhibited sphere-formation by GSCs, with similar efficacy (D). **(II)**. CLR1404 pre-treatment of GSCs for 24 hrs suppressed GBM growth (B) compared to vehicle controls (A) in an orthotopic mouse model, and significantly increased survival time (Control: 59±6.1 days; CLR1404: 94±4.4 days) (C). **(III)**. Mice harboring GSC-derived GBM xenografts were treated with CLR1501 for 24 hours prior to sacrifice and ex vivo isolation of GBM cells into a sphere-forming assay. CLR1501 treatment inhibited sphere formation in the 22 GSC line (B) compared to untreated mice (A and C). CLR1501 effects on a more diffuse GBM derived from 105 GSCs demonstrated more modest effects, suggesting blood-brain barrier properties may play a role in CLR1404 analog efficacy. **(IV)**. 24 hr CLR1404 treatment inhibited AKT activation as evidenced by immunoblotting. Taken together, these data demonstrate CLR1404's therapeutic potential against GBM and its GSCs.

Conclusions and Future Directions

- **The novel phospholipid ether CLR1404 and its analogs specifically label and are retained long-term in both glioblastoma multiforme (GBM) and GBM cancer stem cells (GSCs)**
- **CLR1404 analogs inhibit GBM and GSC cell proliferation in vitro and in vivo, and suppress GBM growth and improve survival in orthotopic mouse models**
- **CLR1404 analogs at least partially exert therapeutic benefit via inhibition of AKT activation, a major molecular hub for oncogenic growth and survival. Analysis of other potential molecular mechanisms is currently underway.**
- **CLR1404's therapeutic potential against GBM and its GSCs, combined with previously demonstrated tumor cell targeting specificity of CLR1404 and its analogs, provides strong evidence for the potential of novel CLR1404-based therapies to improve GBM outcomes.**

Acknowledgements

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