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Semapimod Sensitizes Glioblastoma Tumors to Ionizing Radiation by Targeting Microglia

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Abstract

Glioblastoma is the most malignant and lethal form of astrocytoma, with patients having a median survival time of approximately 15 months with current therapeutic modalities. It is therefore important to identify novel therapies. There is mounting evidence that microglia (specialized brain-resident macrophages) play a significant role in the development and progression of glioblastoma tumors. In this paper we show that microglia, in addition to stimulating glioblastoma cell invasion, also promote glioblastoma cell proliferation and resistance to ionizing radiation in vitro. We found that semapimod, a drug that selectively interferes with the function of macrophages and microglia, potently inhibits microglia-stimulated GL261 invasion, without affecting serum-stimulated glioblastoma cell invasion. Semapimod also inhibits microglia-stimulated resistance of glioblastoma cells to radiation, but has no significant effect on microglia-stimulated glioblastoma cell proliferation. We also found that intracranially administered semapimod strongly increases the survival of GL261 tumor-bearing animals in combination with radiation, but has no significant benefit in the absence of radiation. In conclusion, our observations indicate that semapimod sensitizes glioblastoma tumors to ionizing radiation by targeting microglia and/or infiltrating macrophages.


Competing Interests: The authors have submitted a US provisional patent application 50425-432 (Yousef Al- Abed, Marc Symons and Ian Miller): Method for treating glioblastomas. This application, however, does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Glioblastoma Multiforme (GBM) is the most malignant and lethal form of astrocytoma (WHO grade IV). Despite recent advancements in the standards of care, the outlook for GBM patients remains bleak and new therapies are therefore swiftly required. Currently, the standard of care is gross tumor resection followed by radiation treatment and concurrent chemotherapy [1]. However, even after extensive therapy, relapse is certain and the disease remains lethal, with a life expectancy of little over one year after diagnosis [2].

Microglia are the resident macrophages of the central nervous system (CNS) [3,4]. They are glial cells that are of hematopoietic origin. Microglia are the main phagocytic and immunocompetent cells in the CNS. They are activated by damage or infection and phagocytose debris and other cells. They also present antigens and secrete cytokines that regulate inflammatory responses. Microglia are attracted by glial factors that promote immunosuppression, and glioblastoma cell proliferation, invasion and angiogenesis. Importantly, selective ablation of microglia has been shown to inhibit glioblastoma invasiveness and growth [11,12].

Semapimod (initially termed CNI-1495) is a multivalent guanhydrazone small molecule that was developed as a targeted inhibitor of cytokine-inducible L-arginine transport in macrophages [13,14]. Remarkably, no significant effect of semapimod on cells from other lineages has been noted [15]. Semapimod has been shown to have significant efficacy in several animal models of inflammation, including lethal sepsis [16]. Importantly, semapimod has already been tested in a phase II clinical trial for Crohn’s disease and was shown to be very well tolerated in humans [17]. In this study, we examined whether semapimod can blunt the stimulatory effect of microglia on the malignant behavior of glioblastoma cells, both in vitro and in a syngeneic orthotopic mouse model of glioblastoma.
Materials and Methods

Ethics Statement
All procedures involving mice were conducted in accordance with the National Institutes of Health regulations and ARRIVE guidelines concerning the use and care of experimental animals. The study of mice was approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute. All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Cell Culture
Murine GL261 glioblastoma cells were obtained from the National Cancer Institute (Frederick, MD, USA) and normal murine microglia, isolated from C57Bl/6 mice [18], were obtained from S. Coniglio (Albert Einstein College of Medicine) [19]. Both cultures were maintained in Macrophage Serum-Free Medium (MSFM; Life Technologies Corporation) with 10% fetal calf serum. Microglia were supplemented with 10 ng/mL recombinant mouse granulocyte macrophage–colony-stimulating factor (GM-CSF) (R&D Systems). All cultures were grown at 37°C in a humidified atmosphere of 5% CO2, 95% air. Cell lines were tested for the presence of contaminating mycoplasma during experimentation.

Reagents
Semapimod was produced in house by Dr. Yousef Al-Abed and prepared as a stock concentration of 20 mM in 7% DMSO and ddH2O. It was diluted for experiments using Dulbecco’s phosphate-buffered saline (PBS) to concentrations required.

Invasion Assays
Glioblastoma and microglial cells were labelled with cell tracker green CMFDA and with cell tracker red CMTPX, respectively, and then embedded in 50 μL of 10 mg/mL basement membrane extract (BME) ( Trevigen). The mixture was then placed in a transwell insert (previously coated with 1 μg/mL fibronectin on the bottom side of the 8 μm filter to maintain adhesion of the cells that invaded through the filter) and allowed to polymerize for 30 min at 37°C. Subsequently, serum free medium was added to both wells. To maintain constant cell numbers, cells were plated at a density per invasion chamber of 15 x 104 GL261 cells and 5 x 104 microglia cells in MSFM. Semapimod or its diluent was added at varying concentrations into the BME and in the medium above and below the transwell. Invasion chambers were incubated for 48 h and subsequently fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS). The gel in the transwell inserts was carefully removed. Invaded glioblastoma cells were imaged with a Zeiss Axiovision inverted microscope and a 10 x objective. All invaded cells were counted.

To measure the invasion of microglia toward glioblastoma cells in vitro, we used a variant of the glioblastoma cell invasion assay. First, 15 x 104 GL261 cells were plated in MSFM medium overnight and subsequently were placed in serum free medium. Subsequently, 5 x 104 microglia cells were embedded in 50 μL of 10 mg/mL BME with or without 200 nM semapimod, placed in a 8 μm transwell (previously coated with 1 μg/mL fibronectin on the bottom side of the 8 μm filter to maintain adhesion of the cells that invaded through the filter) and allowed to polymerize for 30 min at 37°C. The transwell chambers were then placed above the previously plated GL261. After 48 h of incubation, the chambers were fixed in 3.7% formaldehyde in PBS. The cells were stained with 0.2% crystal violet in 2% ethanol. The remaining BME was carefully removed and the inserts were allowed to dry.

Cells attached to the bottom of the filter were imaged as for the invasion assay and the total number of invaded microglia was determined.

Colony Formation Assay
GL261 cells and microglia were labeled as described for the invasion assay and were co-cultured in serum overnight and then changed to serum free medium for 48 h in the absence of serum at a 1:1 ratio at a density of 3 x 104 per well of a 6-well plate in the presence or absence of 200 nM semapimod. Subsequently, cells were subjected to 3 Gy of X-ray irradiation using a Radionics 160 kV irradiator. Two days after irradiation, cells were trypsinized, counted using a fluorescence microscope and 300 GL261 cells were plated in 6 cm dishes containing 10% FBS MSFM medium. Colony formation was allowed to proceed for 12 days, with medium changes every other day. Subsequently, the cells were fixed in 3.7% formaldehyde in PBS and stained with 0.2% (w/v) sulphorhodamine B (SRB) dye in 1% acetic acid for 20 min. The dishes were washed with 1% glacial acetic acid and allowed to dry. Plates were scanned and processed using Photoshop 5 (Adobe). Images were changed to grayscale and background threshold intensity was set. Colonies were counted automatically by Image J ( http://imagej.nih.gov/ij/) and the numbers graphed.

Proliferation Assay
50,000 GL261 cells were cultured overnight in the absence or presence of semapimod (50,000 or 150,000) in serum-containing medium and subsequently cultured in the presence or absence of 200 nM semapimod in a 6-well dish in serum-free conditions. Microglia were added in a ratio of 1:1 or 1:3 GL261:microglia. After 72 h of incubation, plates were washed in PBS and fixed in 3.7% formaldehyde in PBS. In initial experiments, we stained the microglia with tomato lectin for 45 mins and stained all nuclei with DAPI. Ten fluorescence micrographs of each culture were taken with a Zeiss Axiowert-based imaging system. Counting of the cells showed that the number of the microglia did not change over the observation period of 3 days. Therefore in subsequent experiments the cells were stained with SRB as described for the colony formation assay. After drying, the plates were de-stained with 500 μL of 10 mM Tris base (Sigma). Eluted SRB was measured by absorbance at 490 nm. To determine the cell growth, the OD at day 1 was subtracted from the OD at day 3.

Animal Experiments
First, 8 week old male C57BL/6J mice were inoculated in the right caudate putamen with GL261 cells. Briefly, animals were placed in a stereotactic frame after pre-anesthesia exposure in a box of 5% isoflurane. Deep anesthesia was maintained on the frame at approximately 2% isoflurane and 2% O2. A burr hole was drilled 1 mm anterior and 2.5 mm to the right of the bregma. With the aid of the stereotactic frame, GL261 cells (2 x 106 cells suspended in 1 μL) were injected with a Hamilton syringe at a depth of 3 mm over a one minute time period. Subsequently, the syringe was left in place for 1 minute to prevent reflux.

Seven days after implantation of the GL261 cells, an osmotic pump (Alzet, Durect) filled with either 0.42 mg/ml solution of semapimod or vehicle was implanted in a subcutaneous pocket on the dorsal flank of the animal. A catheter with attached cannula delivered the drug intracranially over a period of 2 weeks, at a steady rate of 0.25 μl/h, which is equivalent to a dose of 6 mg/kg/day, assuming no blood-brain barrier permeability of the drug. Starting one day after pump implantation, animals were given 2 Gy whole brain irradiation every other day over a period of 10 days (10 Gy total). Animals that lost greater than 20% in body weight during the course of treatment were excluded from the study.
Semapimod inhibits microglia-stimulated glioblastoma cell invasion

In order to examine the effect of microglia on the malignant properties of glioblastoma cells, we used GL261 murine glioblastoma cells and syngeneic microglia isolated from C57Bl/6 mice, a model system that has been extensively used to study the reciprocal interaction between glioblastoma cells and microglia [11,12,19].

A defining feature of malignant glioblastoma is the diffuse invasion of tumor cells into the surrounding parenchyma and microglia have been shown to strongly promote this activity [11,19–22]. To determine the effect of microglia on the invasive properties of glioblastoma cells, we designed a novel 3-dimensional microglia–glioblastoma co-culture assay that provides a better approximation of the in vivo setting than a 2-dimensional configuration. Fluorescently labeled cells were embedded in reconstituted extracellular matrix (BME) in the absence of added serum at a ratio of 3:1 glioblastoma cells to microglia and subsequently placed in transwells provided with an 8 μm pore filter. Using this assay, we observed that microglia stimulate GL261 invasion up to 5 fold and that semapimod interferes with this effect with an IC50 of less than 50 nM (Fig. 1A), similar to the IC50 of semapimod in inflammatory cytokine release from macrophages [14]. Importantly, semapimod, even at a concentration of 10 μM, does not affect serum-stimulated GL261 invasion, underlining the selectivity of semapimod for cells from the monocytic lineage (Fig. 1B).
Semapimod inhibits glioblastoma-induced microglia invasion in vitro

Microglia extensively infiltrate glial tumors [6,8,9]. To examine whether semapimod can also inhibit migration of microglia towards glioblastoma cells, we established an in vitro transwell invasion assay by measuring the number of microglia that invade through a 3-dimensional extracellular matrix toward glioblastoma cells. We observed that the presence of GL261 cells in the bottom well strongly stimulates microglia invasion, by approximately 12 fold (Fig. 1C). This stimulatory effect is abolished by semapimod, with an IC50 of approximately 60 nM.

Semapimod inhibits microglia-stimulated glioblastoma cell survival

A critical problem of malignant glioblastoma is its strong resistance to ionizing radiation (IR) and other therapeutic modalities [23]. The role of microglia in glioblastoma cell survival has not been studied thus far. We therefore examined whether microglia can enhance the survival of GL261 cells after IR and whether semapimod interferes with this function. GL261 cells were plated either in the presence or absence of microglia with or without semapimod (200 nM) for 2 days, followed by radiation treatment (3 Gy). Two days later, glioblastoma cell viability was assayed using a standard colony formation assay. We observed that co-culturing GL261 cells with microglia in the absence of radiation slightly, but significantly, stimulates their survival and that semapimod abolishes this (Fig. 2A). In the presence of radiation, the stimulatory effect of microglia on glioblastoma cell survival is more marked (50%) and this effect is also abolished by semapimod (Fig. 2B). In line with the selectivity of semapimod for cells from the monocytic lineage, semapimod does not affect the survival potential of glioblastoma cell monocultures. Thus, taken together, these data indicate that semapimod inhibits microglia-stimulated glioblastoma cell survival by modulating the activation state of the microglia.

Semapimod does not affect microglia-stimulated glioblastoma cell proliferation

Microglia have been shown to slightly stimulate glioblastoma cell proliferation and therefore we also wanted to examine whether semapimod inhibits this effect. Fig. 3 shows that microglia stimulate GL261 cell proliferation in a fashion that depends on the microglia-glioblastoma cell ratio. However, semapimod (200 nM) does not affect the microglia-stimulated glioblastoma cell proliferation.

Semapimod strongly inhibits tumor cell invasion in vivo

To evaluate the effect of semapimod on the malignant behavior of glioblastoma in vivo, we used orthotopic implantation of GL261 cells into syngeneic C57Bl/6 mice. This model displays all the pathological hallmarks of glioblastoma and is often used for examining the role of microglia in glioblastomagenesis and for preclinical evaluation of immunomodulatory therapies [11,12,19,24].

In order to identify an effective dose of semapimod, we performed pilot studies and chose the minimal concentration that maximally inhibited tumor invasiveness one week after start of treatment. Animals were inoculated with $2 \times 10^4$ GL261 cells into the right caudate putamen. To deliver semapimod, we used an osmotic pump that was implanted subcutaneously in the dorsal flank of the animal and fed a transcranial cannula that was inserted into the tumor. In order to mimic a therapeutic setting, tumors were allowed to develop for 1 week before the onset of
Iba1 staining. (D) Quantification of Iba1 microglia into GL261 tumors. Activated microglia were visualized using student’s 2 tailed t-test. (C) Micrographs illustrating infiltration of microglia in vivo represent the average SEM of 5 different tumors. **: p<0.01

As our in vivo observations showed that semapimod strongly inhibits invasion of microglia toward GL261 cells, we also examined tumor sections for the infiltration of microglia using Iba1 staining (Fig. 4C). No significant difference in the number of microglia per tumor area could be detected, however (Fig. 4D).

The most likely interpretation for this finding is that semapimod treatment was started 7 days after the implantation of the tumor cells, a time at which microglia infiltration already has occurred [25].

As semapimod does not affect GL261 cell proliferation in vitro, we were surprised by the finding that semapimod treatment causes a robust inhibition of tumor size, two weeks after tumor cell inoculation (Fig. 5A). Interestingly, the treated tumors also display a small but significant increase in tumor cell density (Fig. 5B), possibly caused by the strong inhibitory effect of semapimod on tumor cell invasion. Still, the estimated total number of cells in semapimod-treated tumors is significantly less than that in control tumors, indicating that semapimod indeed diminishes the proliferation potential of the tumor cells in vivo (Fig. 5C). The discrepancy between the in vitro and the in vivo tumor cell proliferation data may be due to the fact that the in vivo observations reflect the cumulative effect of many cell divisions, thereby possibly amplifying small differences in cell proliferation that are not detectable in the in vitro experiments.

**Semapimod strongly enhances the therapeutic efficacy of ionizing radiation in a syngeneic orthotopic mouse model of glioblastoma**

To determine the effect of semapimod on the survival of glioblastoma-bearing mice and to examine the effect of semapimod on radiation resistance in vivo, we randomized 4 groups of animals to receive semapimod or diluent in the absence or presence of 10 Gy fractionated whole brain irradiation. We observed that in the absence of radiation, semapimod does not significantly prolong survival (median survival of 22 days for semapimod-treated animals versus 20 days for controls) (Fig. 6). However, whereas radiation alone increased median survival by 12 days, most of the irradiated animals that were treated with semapimod survived at least 40 days beyond the median survival time of control animals and had no detectable tumors as judged by histological analysis (data not shown). These observations indicate that semapimod strongly sensitizes GL261 tumors to radiation.

To examine whether semapimod promotes radiotherapy-induced glioma cell death in vivo, we performed TUNEL staining on sections from control and treated tumors. Treatment with semapimod approximately doubled the percentage of apoptotic tumor cells in irradiated but not in non-irradiated animals (Fig. 7), strongly supporting the hypothesis that semapimod acts as a radiosensitizer in glioma tumors in vivo.

**Discussion**

In this paper, we show that the immunomodulatory drug semapimod markedly enhances the survival of glioblastoma-bearing mice in conjunction with radiation therapy, but not as monotherapy. In addition, our in vitro observations show that semapimod inhibits the survival of irradiated glioblastoma cells in the presence, but not in the absence, of microglia. Taken together therefore, our results strongly suggest that semapimod sensitizes glioblastoma tumors to ionizing radiation by targeting microglia.

The notion that semapimod targets microglia, but not glioblastoma cells, is in line with previous data showing that semapimod is selective for cells derived from the monocytic lineage, i.e. microglia, macrophages and dendritic cells, but not T cells [15,26]. We note that gliomas also are infiltrated by peripheral macrophages [23], which also may promote therapeutic resistance. Thus, the inhibitory effect of semapimod on the
The activation status of infiltrating macrophages may also contribute to the therapeutic effect of semapimod. The mechanism of action of semapimod remains to be elucidated. The only known molecular target of semapimod to date is c-Raf [27]. However, we found that ERK activation in microglia that are stimulated by GL261-conditioned medium is not inhibited by semapimod (data not shown), suggesting that this proposed mechanism is not relevant for our observations. Exactly how semapimod modulates microglial function is currently under investigation in our laboratory.

We note that semapimod has been extensively used as an anti-inflammatory agent [27,28]. Thus, our results that semapimod sensitizes tumors to radiation therapy may seem paradoxical, as the immune system, in general, is thought to counteract tumor formation. We note, however, that there is now mounting evidence in the literature that targeting microglia/macrophages may be of therapeutic benefit in the treatment of glioblastoma [5,6,7,11,12,22,25,29] and other malignancies [30].

Hitherto, the role of microglia in the malignant behavior of glioblastoma cells has largely focused on the invasiveness of the tumor cells [5,6] and the effect of microglia on the survival properties of glioblastoma cells has not been investigated. In this paper, we report that microglia exert a small, but significant, stimulatory effect on the survival of glioblastoma cells that are challenged by ionizing radiation and that semapimod inhibits this effect. In line with this result, we find that semapimod treatment increases the number of apoptotic tumor cells induced by ionizing radiation in vivo and markedly extends the survival of glioblastoma-bearing mice in conjunction with ionizing radiation.

Figure 5. Semapimod inhibits tumor growth in vivo. GL261 cells were implanted and mice were treated as described in Fig. 4. (A) Quantification of tumor volume. Data shown represent the average +/- SEM of 5 different tumors. (B) Quantification of tumor cell density. Data shown represent the average +/- SEM of 5 different tumors. (C) Quantification of total tumor cell number. Data shown represent the average +/- SEM of 5 different tumors. *: p<0.05 student’s 2 tailed t-test.

Figure 6. Semapimod increases survival of glioblastoma-bearing mice in conjunction with ionizing radiation. C57Bl/6 mice were injected orthotopically with GL261 cells. Starting 7 days after cell inoculation, the mice were treated intracranially for 2 weeks with semapimod, delivered via an osmotic pump. Starting on day 8, animals were given 2 Gy whole brain irradiation every other day over a period of 10 days (10 Gy total). *: p<0.05. Chi square test, with 3 degrees of freedom.

Figure 7. Semapimod increases apoptosis induced by radiation in vivo. GL261 tumors were generated in mice and treatments were carried out as described in Fig. 6, except that the total radiation was 8 Gy. The mice were euthanized the day after the last radiation treatment. Frozen brain sections were analyzed for apoptosis using TUNEL staining as described in Materials and Methods. (A) Micrographs of tumor sections illustrating TUNEL staining. (B) Quantification of TUNEL staining. The percentage of apoptotic cells was calculated using 20–60 micrographs per tumor section. Data shown represent the average +/- SEM of 3–4 different tumors. *: p<0.05 student’s 2 tailed t-test.
bears animals that are treated with radiation. The relatively large effect size of semapimod treatment observed in vivo may be explained by the use of a fractionated radiation regimen (5 doses of 2 Gy, every other day), which is expected to magnify the survival benefit seen in the single dose of irradiation in the in vitro setting. The striking enhancement by semapimod of the therapeutic effect of ionizing radiation in glioblastoma-bearing mice is in line with the radiosensitization observed by depleting macrophages in a subcutaneous melanoma model [31] and the marked enhancement of the inhibitory effect of paclitaxel on mammary tumor pulmonary metastasis caused by a CSF-1R inhibitor that blocks macrophage recruitment to the tumor [32]. Notably, in all these cases, targeting the tumor-associated microglia/macrophage compartment on its own showed little or no therapeutic benefit. These observations strongly suggest that the microglia/macrophage compartment plays a critical role in therapeutic resistance and that targeting this compartment in combination with other therapeutic modalities is likely to be of significant clinical benefit. We found that semapimod has a marked inhibitory effect on glioblastoma tumor cell invasion both in vitro and in vivo. These observations are in line with previous reports that have demonstrated that interference with the function of microglia or depletion of the microglial compartment has a strong inhibitory effect on glioblastoma cell invasion [11,19]. Interestingly however, the marked inhibition in tumor invasiveness caused by semapimod is not accompanied by a significant increase in survival of glioblastoma-bearing mice. This observation suggests that blocking invasion on its own may have little therapeutic benefit, at least in the setting that we have explored. However, in other preclinical settings, inhibiting invasion could yield therapeutic benefit. Possible scenarios include the implementation of a tumor resection model [33], which better mimics current therapeutic approaches in the clinic, or combined treatment with bevacizumab, which has been shown to enhance the invasive behavior of glioblastoma tumors [34,35].

An attractive feature of semapimod is that it is an investigational drug that already has been tested in several clinical trials [17,36,37]. Importantly, semapimod was shown to be very well tolerated in humans and displays a good safety profile. Thus, our observations suggest the utility of repositioning semapimod as an immunomodulator for the treatment of glioblastoma, as up-front adjuvant to standard therapy, concurrent with radiation and temozolomide chemotherapy [1].

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Author Contributions

Conceived and designed the experiments: YM YA MS. Performed the experiments: YM SD DWM THT MI RR. Analyzed the data: YM RR MS. Contributed reagents/materials/analysis tools: YA. Wrote the paper: YM.

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Glioblastoma Radio-Sensitization by Semapimod