CA-4948 For the Treatment of Melanoma Brain Metastasis

Bently Doonan[1,2], Christina Von Roemeling[2], Kelena Klippel[2], Lan Hoang-Minh[2], and Duane Mitchell[2]

[1.] University of Florida Health Cancer Center. [2.] University of Florida Brain Tumor Immunotherapy Program.

BACKGROUND

Melanoma brain metastases (MBM) remain the primary driver for melanoma associated mortality and morbidity. It is estimated that 40-60% of patients with metastatic melanoma will develop MBM during the course of their disease [1]. With treatment, local control of MBM is obtained in many patients but even with combination immunotherapy and stereotactic radiosurgery (SRS), 50% of patients will experience an intracranial relapse within the first year of their disease [2]. Novel strategies are desperately needed for this patient population. An ideal therapeutic would work in concert with current therapy, have strong blood brain barrier penetration, and have the potential to work in both BRAF mutated and BRAF wildtype patients. Our group has identified IRAK-4 as a novel target in MBM and proposes the oral IRAK-4 inhibitor CA-4948 as potential treatment in MBM in combination with aPD1 therapy.

Melanoma represents an inflamed and immunologically active tumor with upregulation of multiple mechanisms for growth, proliferation, angiogenesis, metastatic seeding, and resistance to therapy [3]. One of these mechanisms that utilizes inflammatory signaling to promote progression is the Toll-like receptor (TLR) signaling cascade. TLRs are an aspect of the innate immune system designed to recognize microorganisms and signals released from dying cells [4]. This innate activation triggers downstream adapter proteins like myeloid differentiation primary response protein 88 (MyD88) and interleukin (IL)-1 receptor-associated kinase (IRAK-1 and -4) which activates transcription of multiple cellular kinases and transcription factors (JNK, AP-1, MAPK, and NF-κB) upregulating production of inflammatory chemokines and cytokines like TNF-α, IL-1, IL-6, and IL-8 [4, 5]. IRAK-4 also upregulates expression of proteins involved in cell survival and division [4]. These signals work to recruit adaptive immune cells to the environment and promotes sterile inflammation [5]. While this is initially a positive feature leading to pathogen clearance, unchecked and rampant upregulation of this inflammatory cascade leads to tumor growth and proliferation and reflexive suppression of the immune system. Studies in melanoma have shown IRAK-4 is upregulated and activated in most cutaneous melanoma samples [6, 7]. IRAK-4 is also capable of promoting carcinogenesis regardless of mutation status [8]. In previous studies, inhibition of IRAK-4 directly inhibited growth of melanoma in a xenograft mouse model of melanoma, and worked synergistically with cytotoxic chemotherapy [4]. Increased expression of IRAK-4 in melanoma has also been shown to contribute to increased numbers of phenotypically exhausted cytotoxic T cells, myeloid derived suppressor cells (MDSCs), and T regulatory cells [6]. MyD88/IRAK-4 has also been implicated in upregulation of PD-1 expression on tumor associated macrophages (TAMs) sustaining their immunosuppressive functioning in melanoma [5]. Further, inhibition of MyD88/IRAK-4 promoted an increase in CD8+ T cell recruitment to the tumor site, reduced expression of suppressive TAMs and improved the efficacy of aPD1 therapy in a mouse model of melanoma [5]. For all of these reasons, we propose targeting IRAK-4 may work in combination with aPD1 therapy in MBM patients.

METHODS

To test this hypothesis we first defined the expression of IRAK-4 and its downstream effect in human MBM. Human MBM samples (MM3042, MM3712, and MM3088) were subjected to cryosectioning and advanced immunohistochemistry looking for IRAK-4, IRAK-1 (co-chaperone molecule), and NF-KB expression **(Figure 1).** We additionally counter stained for immune cells within the tumor microenvironment (CD45) and astrocytes (GFAP). Next, we examined the protein expression of NF-KB pathway proteins in human MBM samples (MM202685, MM228917) and normal brain control using a proteomic profiling assay (R&D systems) **(Figure 2).** We next validated the CNS applicability of an oral IRAK-4 inhibitor, CA-4948 (Emavusertib®, Curis Inc) in both naïve and tumor bearing mouse models through advanced mass spectroscopy in collaboration with the UF Mass Spectroscopy Core. In these experiments, naïve or tumor bearing BALB/c mice were treated with CA-4948 daily and mice were euthanized at pre-planned time points. Mouse serum, brain, and CSF were collected at these time points and analyzed via mass spectroscopy **(Figure 3).** Next, we performed in vivo determination of CA-4948 activity in dual tumor implantation models of metastatic melanoma. In these experiments, C57BL6 mice were implanted with both subcutaneous (D-3) and intracranial (D0) B16.F10 melanoma cells to establish tumors. Dual implantation models were chosen as previous work has shown that in mouse models of MBM, the effect of immune checkpoint inhibition is dependent the presence of both intracranial and extracranial disease [9]. Tumors were allowed to grow until D5 then treated with single agent CA-4948 (50mg/kg or 100mg/kg) or excipient control for a duration of 14 days. Mice were observed for survival and analyzed using a logrank trend for significance. A pre-planned cohort was euthanized at day 7 of treatment and intracranial tumor volumes were compared between excipient and CA-4948 100mg/kg treatment arms **(Figure 4).** Next, using the same treatment paradigm we treated C57BL6 mice with CA-4948 or excipient control for 5 days following dual tumor implantation. Mice were euthanized and their brains harvested and subjected to tissue clearing via CLARITY method. Advanced immunohistochemistry was then performed on cryosectioned tissue looking for changes in expression of NF-KB, pERK, and pP38 (MAPK) **(Figure 5).** Finally, using the same dual tumor implantation model, C57BL6 mice were implanted with B16.F10 cells and subjected to treatment with CA-4948, aPD1, CA-4948 + aPD1, or control (IgG + excipient) for two weeks. Subcutaneous flank tumor volumes were then measured in 3 dimensions using electronic calipers and tumor volume plotted **(Figure 6).**

RESULTS

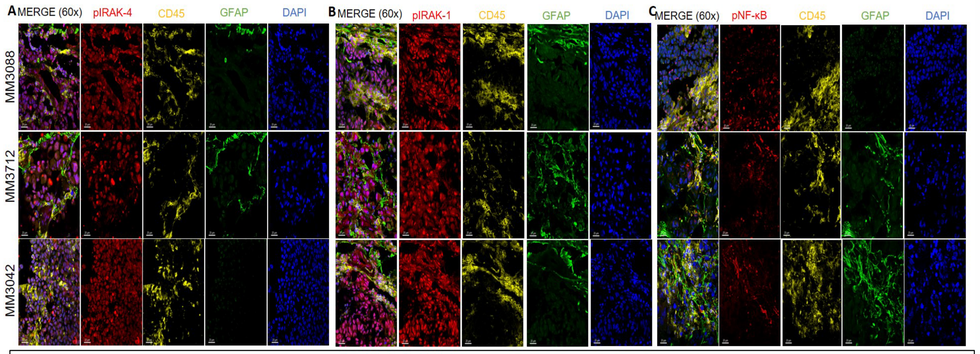


Figure 1: Advanced immunohistochemistry and confocal microscopy of human melanoma brain metastases samples (MM3088, MM3712, MM3042). (A) MBM have strong IRAK-4 expression, immune cells of the tumor microenvironment counterstained with CD45 and astrocytes stained with GFAP, DAPI used as nuclear localization stain. Merge panel shows upregulated expression of IRAK-4 in both tumor and tumor microenvironment at 60x magnification. (B) MBM additionally expression high levels of phospho-IRAK-1 with reduced levels in surrounding tumor microenvironment. (C)MBM have upregulated expression of active NF-κB which is main transcriptional target of IRAK-4.

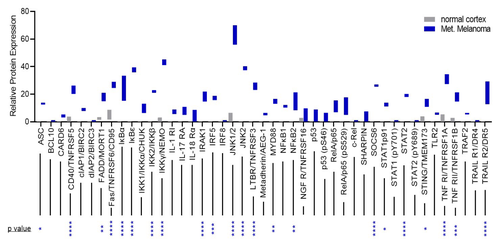


Figure 2. Proteomic evaluation of NF-KB pathway in MBM vs normal brain tissue. Human MBM samples (MBM202685, MBM228917) and normal brain tissue were subjected to enzymatic digestion and protein analysis to ensure equal volume loading. Samples were then analyzed for NF-KB pathway proteins using R&D systems Proteome Profiler per protocol. Data representative as aggregate of two samples per type. Significance determined by 2-way Anova, multiple comparisons (column means) *p<0.05.

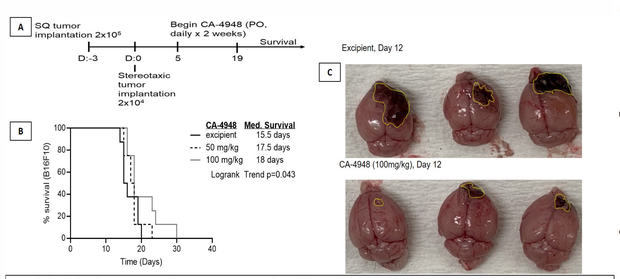
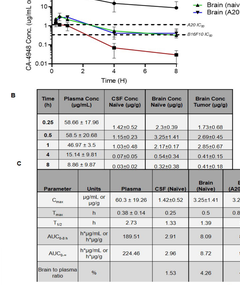


Figure 4: Drug treatment and survival analysis in dual subcutaneous and intracranial implantation model of metastatic melanoma using B16.F10 cells in C57Bl/6 mice. (A) Schematic representation of dual implantation model. Mice are implanted with 2x10^5 B16.F10 cells subcutaneously into the right hind flank on day -3. Mice then undergo stereotactic implantation of cells into right cerebral hemisphere as previously described on day 0. Tumors are allowed to establish for 5 days and mice were then treated with oral CA-4948 at dose of 50mg/kg, 100mg/kg, or excipient control for 2 week treatment course. (B) Kaplan-meyer plot of overall survival, there is a trend towards significant improvement in survival in dual implantation model similar to that observed with intracranial alone (n=10 mice per group). (C) Planned subgroup analysis of intracranial tumor size in excipient vs 100mg/kg treated mice following 7 days of treatment. Intracranial tumor volume is significantly reduced in mice treated with CA-4948 vs control (n=3 mice per group).



Time (hr)	Plasma Conc (ug/mL)	CSF Conc Naïve (ug/g)	Brain Conc Naïve (ug/g)	Brain Conc Tumor (ug/g)
0.25	58.66 ± 17.96	1.42±0.52	2.3±0.39	1.73±0.68
0.5	58.5 ± 20.68	1.15±0.23	3.25±1.41	2.69±0.45
1	46.97 ± 3.5	1.03±0.48	2.17±0.17	2.85±0.67
4	15.14 ± 9.81	0.07±0.05	0.54±0.34	0.41±0.15
8	8.86 ± 9.87	0.03±0.02	0.32±0.38	0.41±0.18

Parameter	Units	Plasma	CSF (Naïve)	Brain (Naïve)	Brain (A20 Tumor)
C_{max}	µg/mL or µg/g	60.3 ± 19.26	1.42±0.52	3.25±1.41	3.22±0.18
T_{max}	h	0.38 ± 0.14	0.25	0.5	0.83±0.29
$T_{1/2}$	h	2.73	1.33	1.39	1.19
AUC_{0-8}	h*µg/mL or h*µg/g	189.51	2.91	8.09	8.68
$AUC_{0-\infty}$	h*µg/mL or h*µg/g	224.46	2.96	8.72	9.39
Brain to plasma ratio	%		1.53	4.26	4.95

Figure 3. Plasma, brain, and CSF concentration profiles. Profiles were determined following a single oral dose of CA-4948 at 150mg/kg administered as 0.25% w/v hydroxyethyl cellulose, 0.5% v/v Tween 20, and sterile filtered water in 18 BALB/c mice. Animals were euthanized at various time points 0.25, 0.5, 1, 4, 8 h post dose (N=3 per time point). Additional cohort treated with stereotactic implantation of A20 cells to show tumor bearing effect on drug penetrance. In tumor bearing mice samples were collected at D14 following implantation. The UPLC-MS/MS bioanalysis was utilized for data acquisition. A. Time to peak concentrations of oral CA-4948 in plasma, brain parenchyma, and CSF. The IC_{50} for B16F10 melanoma cells over layed as dark dashed line. B Peak drug concentration over time across three compartments. C. pK analysis across the three specified compartments. Abbreviations (Cmax= plasma peak concentration, Tmax= time to reach Cmax, T1/2= elimination half-life, AUC= area under the concentration time curve.)

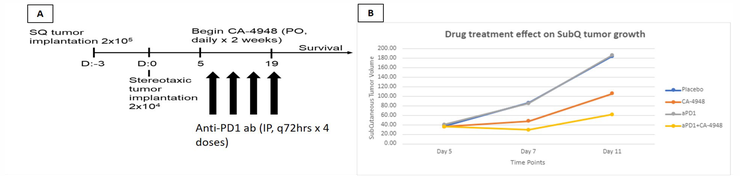


Figure 6. Dual implantation model of combination CA-4948 and aPD1 therapy. (A) Schematic of experimental design. C57Bl/6 mice underwent subcutaneous and intracranial B16.F10 tumor implantation as previously described. Tumors were allowed to establish for 5 days prior to starting therapy. Mice were then treated with either oral CA-4948 100mg/kg qD alone for 2 week treatment course, anti-PD1 ab alone administered IP every 72hrs for total of 4 doses, combination oral CA-4948 and IP anti-PD1 ab, or control (oral excipient and IP IgG). Starting on initial day of treatment subcutaneous flank tumor volumes were measured in three dimension (L x W x Depth) and recorded on days 5, 7, and 11. (B) total tumor volume measurements for mice treated with the 4 planned treatment groups plotted over time. Results show CA-4948 alone and CA-4948 plus anti-PD1 ab treatment significantly slowed and reduced growth of subcutaneous tumors. There is an overall trend towards the combination therapy reducing tumor volume growth velocity to a higher degree than CA-4948 alone and mice were observed in this treatment cohort with tumor regression (n=10 mice per group).

CONCLUSIONS

- IRAK-4 is highly expressed in human MBM and this expression is also seen in the tumor microenvironment and reactive astrocytes
- Upregulated IRAK-4 leads to downstream increases in protein expression of NF-KB, JNK1/2, IRAK-1, and reflexive suppression of inflammation through IKBα, IKBε, and SOCS6
- CA-4948 reaches rapid BBB penetrance and maintains therapeutically significant concentration in the CNS for up to 8 hrs following single oral dose
- CA-4948 significantly reduces intracranial B16.F10 tumor volume in vivo resulting in significant increase in survival compared with control
- CA-4948 treatment reduces the expression of important transcription pathways NF-KB, pERK, and pMAPK in vivo
- CA-4948 suppresses subcutaneous melanoma tumor growth and has combinatorial benefit to growth suppression with aPD1 therapy in aggressive mouse model of melanoma
- Further study on survival benefit to combination CA-4948 in combination with aPD1 is underway and will serve as basis for in human phase I/II clinical trial in development

Figure 5:CA-4948 significantly downregulates expression of pERK, pP38, and pNF-KB in B16.F10 brain metastases. C57BL/6 mice were implanted with B16.F10 cells as previously described and tumors were allowed to establish for 5 days before starting therapy with either oral CA-4948 (100mg/kg qD) or excipient control. After 5 days of treatment mice were euthanized and brains were embedded in hydrogel and subjected to tissue clearing via CLARITY procedure. MBM were then cryosectioned and immunohistochemistry performed staining for pERK **(A)**, pP38 **(B)**, and pNF-KB **(C)**. CD45, GFAP, and DAPI were used as counterstains. Images represent 60x magnification. Imaris® imaging software was then utilized to determine cellular expression of target proteins **(D-F)** compared with total nucleated cells. Data represent 10 independent tissue slices per treatment arm and comparison of values made through two-way ANOVA for determination of significance (p < 0.05).

REFERENCES

1. Liu, H., et al., *Predictive value of a nomogram for melanomas with brain metastases at initial diagnosis.* Cancer Med, 2019. **8**(18): p. 7577-7585.
2. Acharya, S., et al., *Distant intracranial failure in melanoma brain metastases treated with stereotactic radiosurgery in the era of immunotherapy and targeted agents.* Adv Radiat Oncol, 2017. **2**(4): p. 572-580.
3. Doonan, B., J. Hathaway, and A. Haque, *Melanoma Immunomodulation: A War of Attrition*, in *Treatment of Metastatic Melanoma*. 2011, InTech.
4. Srivastava, R., et al., *Augmentation of therapeutic responses in melanoma by inhibition of IRAK-1,-4.* Cancer Res, 2012. **72**(23): p. 6209-16.
5. Tartey, S., et al., *A MyD88/IL1R Axis Regulates PD-1 Expression on Tumor-Associated Macrophages and Sustains Their Immunosuppressive Function in Melanoma.* Cancer Res, 2021. **81**(9): p. 2358-2372.
6. Geng, D., et al., *Induction of IRAK-M in melanoma induces caspase-3 dependent apoptosis by reducing TRAF6 and calpastatin levels.* Commun Biol, 2020. **3**(1): p. 306.
7. Martin Lasola, J.J., et al., *Abstract B31: IRAK-4 signaling in melanoma contributes to reduced expression of inflammatory factors in vitro while contributing to tumor growth in vivo*. 2017, Cancer Immunol Research: Cancer Immunology Research.
8. Jain, A., S. Kaczanowska, and E. Davila, *IL-1 Receptor-Associated Kinase Signaling and Its Role in Inflammation, Cancer Progression, and Therapy Resistance.* Front Immunol, 2014. **5**: p. 553.
9. Taggart, D., et al., *Anti–PD-1/anti–CTLA-4 efficacy in melanoma brain metastases depends on extracranial disease and augmentation of CD8+ T cell trafficking.* Proceedings of the National Academy of Sciences, 2018. **115**(7): p. E1540-E1549.

CONTACT

Bently Doonan, MD, MS
Assistant Professor
University of Florida Health Cancer Center
2033 Mowry Road, Gainesville FL 32610
352-273-9894 • Bently.doonan@medicine.ufl.edu