Astrocyte “Cellular Backpacks” for Targeted Drug Delivery to Melanoma Brain Metastases via Innate Migration

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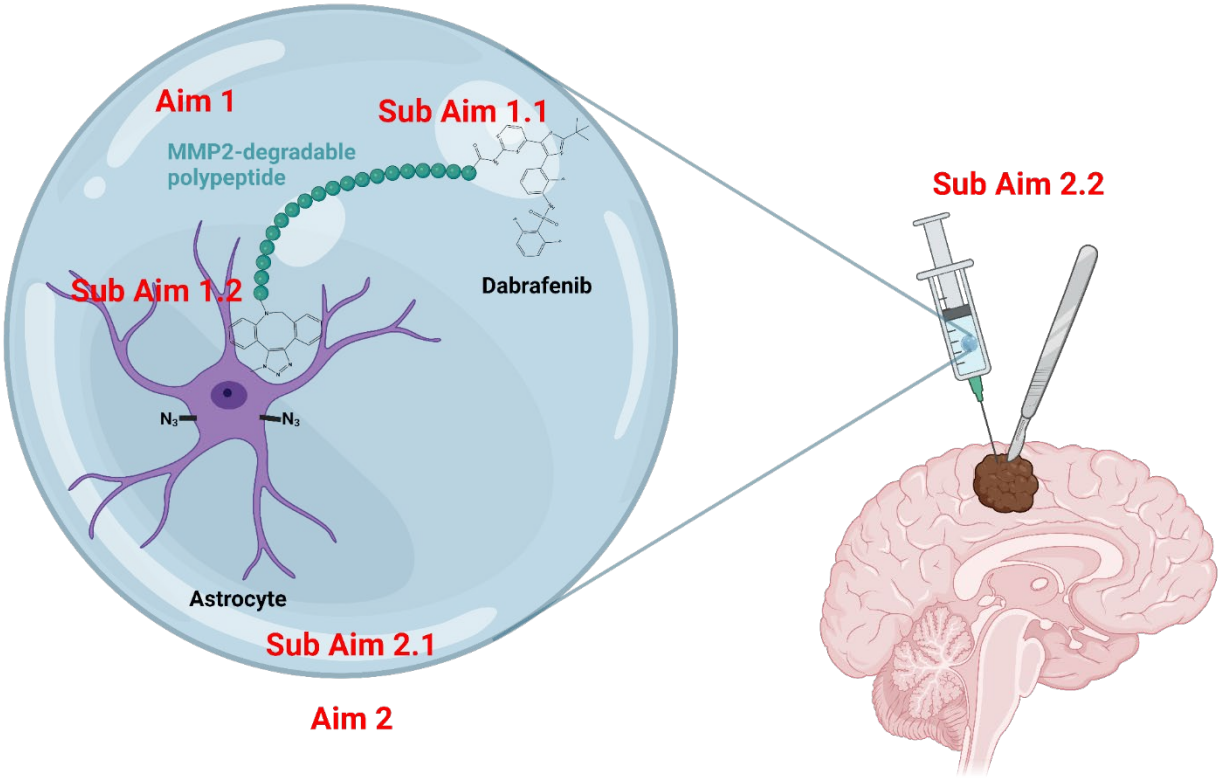
ABSTRACT

Current targeted drugs for melanoma brain metastases (MBM), a pervasive and lethal complication of melanoma, face clinical challenges due to their limited durability. These drugs are administered orally, leading to systemic responses associated with adverse effects and inefficiency due to rapid brain clearance. Within the tumor microenvironment (TME), invasive melanoma cells recruit native astrocytes, which migrate to the tumor periphery, presenting an unexplored avenue for drug delivery. Herein, we hypothesize that **targeted drug delivery to the TME via astrocyte innate migration will improve drug specificity and efficacy while reducing the risk of adverse patient outcomes**. This innovative drug delivery avenue will be developed and evaluated in the following Specific Aims: (1) an MMP-2-degradable polypeptide will couple patient-derived astrocytes with dabrafenib, a representative BRAF inhibitor commonly employed in MBM treatment, forming the "cellular backpacks"; and (2) a pH-responsive microgel will be developed to encapsulate the astrocyte backpacks, protecting them during handling and intracranial injection for in vivo testing after surgical removal of MBM tumor. Within the acidic TME of MBM, the microgel will gradually degrade, releasing the engineered astrocytes, which will be recruited by melanoma cells and migrate intratumorally. In proximity to the MMP-2 secreted by melanoma cells, the polypeptide will be cleaved, releasing the dabrafenib. Through these two levels of niche-responsive conjugation, astrocyte "cellular backpacks" will enhance dabrafenib's delivery specificity, intratumor concentration, half-life, and duration of response while reducing the risk of adverse systemic effects. Overall, this novel pathway offers a promising and innovative strategy for targeted drug delivery and potential improvements in MBM patient outcomes.



Targeted drug delivery via astrocyte migration to melanoma brain metastasis enhances drug specificity while reducing adverse effects.

SPECIFIC AIMS

Skin cancer is the most prevalent type of cancer in the United States[1], and melanoma is the most lethal skin cancer due to its high potential for metastasis[2]. Among the various complications arising from melanoma metastasis, melanoma brain metastases (MBM) are the most common and deadly[3]. In addition to surgery, radiation, and immunotherapy, targeted therapy for MBM has demonstrated significant responses, which is due to metastatic melanomas carrying a substitution mutation resulting in hyperactive BRAF (RAF kinase controlling cell proliferation and differentiation)[4]. BRAF inhibitors, such as dabrafenib, have been extensively developed and are commonly employed in MBM treatment[5]. However, due to the presence of the blood-brain barrier (BBB), oral administration of dabrafenib encounters clinical challenges resulting from limited drug delivery, rapid clearance from the brain, a short half-life, and adverse systemic effects[6,7]. The cranial space subsequently acts as a pharmacologic sanctuary[8], contributing to short duration of responses (DOR), development of drug resistance, and increased risk of relapse.

Recent studies have demonstrated that within the tumor microenvironment (TME), upon brain penetration, melanoma cells recruit astrocytes[9], which are responsible for neuronal repair and support through metabolism regulation and structural maintenance[10,11]. These astrocytes migrate towards the tumor periphery and crosstalk with melanoma cells, facilitating the establishment of melanoma cells within the brain[12]. This phenomenon also offers a promising yet unexplored avenue for drug delivery. Herein, we propose that **targeted drug delivery to the TME facilitated by astrocyte innate migration will enhance drug specificity and efficacy, while reducing the likelihood of adverse patient outcomes**. This proposal will be structured through two **Specific Aims**:

Specific aim 1: Engineer patient-derived astrocytes as dabrafenib-carrying "cellular backpacks"

Metastatic melanoma cells secrete high levels of matrix metalloproteinase-2 (MMP-2) in TME[13]. Consequently, an MMP-2-degradable polypeptide is employed to conjugate astrocytes with dabrafenib, such that dabrafenib remains as pro-drug while being transported by the astrocytes. The release of dabrafenib from the astrocyte occurs concurrently with peptide cleavage as the astrocyte migrates into the TME.

• **Sub aim 1.1**: Conjugate dabrafenib with MMP-2-degradable polypeptide. MMP-2-degradable polypeptide will be purchased and functionalized acid at the C-terminus with the free amine group of dabrafenib via HATU coupling. The N-terminus will be functionalized with dibenzyl cyclooctyne (DBCO) using DMTMM coupling.

• **Sub aim 1.2**: Conjugate patient-derived astrocytes with dabrafenib-loaded MMP-2-degradable polypeptide. Patient-derived induced pluripotent stem cells (iPSC) will be differentiated into astrocytes, which will be metabolically labeled with pendant azides by conditioning the media with an azide-labeled sugar (mannose-N_3). Subsequently, DBCO-peptide-dabrafenib will be conjugated to the azide-bearing astrocytes through strain-promoted azide-alkyne cycloadditions. Following that, the viability and compatibility of the astrocytes will be assessed. The potency and migration ability of the assembled astrocyte backpack will be evaluated through in vitro 2D and 3D co-culture with invasive BRAF-mutated melanoma cells.

Specific aim 2: Encapsulate engineered astrocytes in pH-responsive microgel for in vivo delivery

Engineered astrocytes will be administered via intracranial injection. To protect the astrocyte backpack and to provide structural support, a microgel will be formulated for encapsulating the engineered astrocytes. Given the contrasting pH conditions between the basic cerebrospinal fluid and the acidic TME[14], the microgel will be acid-liable to minimize nonspecific delivery and selectively release the drug-loaded astrocytes near the MBM TME.

• **Sub aim 2.1**: Encapsulate astrocyte backpack into acid-liable microgel. Amine-terminated polyethylene glycol (4arm 20kDa PEG-NH_2) will be conjugated to the carboxylic acid of nitrobenzyl groups via DMTMM coupling. Upon exposure to 405 nm light during photoirradiation, PEG-nitrobenzyl will form an aldehyde, which can subsequently react with excess free amines to form an imine crosslink. This crosslink is hydrolysable at lower pH conditions within the TME, resulting in the gradual release of engineered astrocytes. The tunable moduli, degradation rates, injectability, and rate of cell release will be quantified.

• **Sub aim 2.2**: Evaluate the efficiency of astrocyte backpack in early undetectable MBM and post-surgery tumor removal in vivo. The early stages of MBM are challenging to detect, often resulting in a missed treatment window. In contrast, later stages typically require surgical removal of the tumor, which can leave a cavity in the brain. This aim assesses the sensitivity and surveillance capabilities of the microgel containing astrocyte backpacks in a mouse model with undetectable early-stage MBM. For later stages, a model with the brain tumor surgically removed will receive a direct injection of the microgel into the brain cavity to evaluate its supportive function and its potential in preventing relapses.

RESEARCH STRATEGY

Significance

Melanoma brain metastases (MBM) exhibits high prevalence and high mortality

In the United States, skin cancer is the most prevalent cancer type[15], and melanoma, a subtype of skin cancer, is the fifth most common malignancy[2]. Melanoma accounts for over 80% of skin cancer-related deaths despite representing only 1% of skin cancer cases[2], making it a highly mortal skin cancer due to its significant metastatic potential. Brain metastases are very common in metastatic melanoma with 20-40% of metastatic melanoma patients presenting brain metastases at the time of diagnosis and 50% developing brain metastases post-diagnosed[3]. Patients with MBM has a median survival of only 12.8 months, and the overall mortality rate for MBM is 80-85%[16]. Considering the increasing exposure to UV light, the primary risk factor contributing to cutaneous melanoma, and the fact that 50% of cutaneous melanoma patients will develop MBM[17], the annual incidence and overall prevalence of MBM are expected to increase at a compound annual growth rate of 10.3% from 2023 to 2030[18] with a projected market size of $11.31 billion in 2030[19]. Consequently, MBM represents a significant public health concern that requires attention.

Current MBM treatments have advancement but still face significant clinical challenges

In addition to surgery, radiation, and immunotherapy, targeted therapy has shown significant promise in treating MBM. About 50% of cutaneous melanoma and over 90% of MBM cases carry a BRAF substation mutation that enhances the BRAF activity by 100-200 times[4]. Consequently, BRAF inhibitors (BRAFi) have emerged as the predominant treatment option. Compared to the previous BRAFi for MBM treatment, dabrafenib offers improved attributes, including less adverse effects and a high efficacy in reducing the size of melanoma tumors[5].

However, the current oral administration of dabrafenib[20] still presents several challenges in treating MBM. During the early stages of micrometastases with an intact blood-brain barrier (BBB)[6], orally administrated dabrafenib encounters limited delivery to the intracranial space. This limitation arises from dabrafenib's specific design to prevent BBB penetration, aimed at minimizing side effects on the brain[7]. Once within the brain, dabrafenib faces difficulties in achieving high concentration around or within the tumor, resulting in reduced effectiveness in treating MBM and potential side effects on normal brain tissues, including cerebral hemorrhage and neurotoxicity due to the inhibition of normal BRAF activity[21]. Moreover, dabrafenib is a dual substrate of BCRP and P-gp, which are BBB efflux transporters that actively clears dabrafenib from intracranial space[5]. In the later stages of macrometastases with a disrupted BBB[6], dabrafenib can more easily diffuse out, exacerbating the issue. All these factors contribute to sub-pharmacological concentrations of dabrafenib in the brain, creating a pharmacological sanctuary[8] that selectively promotes the development of drug resistance and later relapse. Some studies have suggested increasing the oral dosage to raise intracranial and intratumoral dabrafenib concentrations[5], but this approach may result in additional systemic adverse effects. Therefore, the development of a more effective delivery strategy to concentrate dabrafenib around and inside MBM tumors is critical.

Astrocytes facilitating MBM adaptation and progression in the brain provides a novel delivery avenue

As the most abundant glial cells in the central nervous system, astrocytes are responsible for repairing and maintaining neurons following brain injuries through metabolic and structural regulation[10]. Additionally, astrocytes contribute to the basic pH of cerebrospinal fluid by supplying bicarbonates, ensuring the normal synaptic transmission between neurons[11]. Intriguingly, during the early stages of MBM, invading melanoma cells recruit and establish direct cell-cell contact with astrocytes through gap junctions[9], facilitating the exchange of molecules. In later stages, astrocytes also infiltrate the tumor[12]. This interaction sustains the melanoma cells' aggressiveness, increases their resistance to chemotherapy, and enhances their proliferative potential[22]. The recruitment, proximity, and intratumor migration of astrocytes make them a highly suitable vehicle for drug delivery. Consequently, this provides a highly promising and innovative avenue for drug delivery that is worth further investigation.

Novel bioconjugation approaches for drug-carrying astrocyte "cellular backpacks"

Recent advances in cell-mediated therapeutic delivery have focused toward utilizing metabolic labeling and innate cellular mechanisms as targeting pathways for addressing the immune system (indirect), such as directing CAR-T cell cytotoxicity, T cell infiltration, and tumor cell accumulation[23]. However, many of these current approaches lack adaptability to the TME of MBM, due to lack of physical accessibility (extravasation

and migration results in multiple, dispersed migratory metastases), and rapid clearance from the TME[6]. However, these limitations can be addressed by utilizing innate astrocytic migration to the TME, coupled with an engineered delivery mechanism (direct). In this investigation, astrocytes will act as a delivery vehicle and "cellular backpacks" will be developed via metabolic labeling, followed by subsequent conjugation of a releasable drug, permitting cell-mediated delivery.

Within the periphery of MBM, bidirectional signaling between astrocytes and melanomas creates a synergistic, reciprocal feedback mechanism, potentiating the invasiveness of melanoma cells[24]. Signaling from metastatic melanoma cells stimulates local astrocytes to upregulate the expression of IL-23, a pro-inflammatory cytokine. Astrocyte-secreted IL-23 subsequently acts as a reciprocating signal, stimulating melanoma cell secretion of MMP-2 in a pro-inflammatory feedback mechanism[9]. This reciprocal signaling functions as a matrix remodeling cascade to permit further extravasation and metastases[24]. This signaling mechanism, however, provides valuable insight regarding the potential for novel cell-mediated drug delivery. Similarly, the locally concentrated proteolytic activity provides a potential chemical cue for TME-specific targeted release. Similarly, the tumor microenvironment and surrounding niche are uniquely characterized by an atypically acidic pH[14]. This irregularity in locally decreased pH provides a second, semiorthogonal cue for TME-specific targeted release.

Although cell-mediated therapeutics have been investigated, it is well known that cellular transplantation via direct injection results in poor cell viability. However, it has been previously reported that cell encapsulation within hydrogels significantly improves viability due to protection from shear stress and extensional flow[25]. Moreover, hydrogel encapsulation for in vivo injection offers a myriad of beneficial factors, including the potential for tuning controlled release and minimizing rejection[25]. More specifically, microfluidic encapsulation in granular hydrogels offers these same factors, with the added benefits of monodisperse interpercolating pore space and increased surface area to volume ratio, permitting rapid diffusion of local biologics as well as resident cells within the native milieu[26]. Similarly, these granular hydrogels can be engineered to control degradation and release rates in response to orthogonal cues (i.e. pH and MMP)[27].

Innovation

Dabrafenib has been employed as a common treatment for MBM through oral administration. However, its efficacy and DOR have been substantially compromised due to limited drug delivery to the cranial space. Our microgel-protected migratable astrocyte backpacks feature two tiers of niche-responsive conjugations: acid-sensitive microgel and MMP-2-responsive linker between astrocyte and dabrafenib. Upon intracranial injection, these conjugations will facilitate the gradual release of the astrocyte backpacks loaded with dabrafenib in proximity to melanoma cells in the MBM tumors, achieving specific drug delivery, elevating intratumor drug concentrations, and extending the dabrafenib half-life. This approach aims to prolong the DOR of dabrafenib while minimizing the risks associated with adverse systemic effects, drug resistance, and relapses. In summary, this innovative pathway holds the potential to introduce a novel strategy for targeted drug delivery and, consequently, may enhance the overall outcomes for MBM patients.

RESEARCH APPROACH

Central hypothesis: Astrocyte cellular backpack enhances the drug delivery both in vitro and in vivo

Specific aim 1: Engineer patient-derived astrocytes as dabrafenib-carrying "cellular backpacks"

Rationale

Upon stimulation by chemokines in the TME, MMP-2 is secreted by brain-metastasizing melanoma cells to increase their invasiveness[9]. Consequently, a polypeptide degradable by MMP-2 is used to conjugate dabrafenib and astrocyte so dabrafenib can be transported by astrocyte into the MBM tumor and released in TME where MMP-2 is overexpressed.

Dabrafenib's low aqueous solubility necessitates the prior conjugation of the peptide to enhance drug solubility, streamline subsequent conjugations, and facilitate drug functionalization. The C-terminus of the peptide will be joined to dabrafenib's free amine via hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) coupling due to its high efficacy and compatibility with various solvents and conditions, which is beneficial for the sparingly soluble dabrafenib. Because the MMP-2-degradable peptide comprises fewer than 25% charged residues, the use of organic solvents, such as DMF, is recommended for the reaction. Additionally, the N-terminus will be linked to dibenzylcyclooctyne (DBCO), and astrocytes will be metabolically

labeled with azide for efficient reagent-free click chemistry[28] to load the dabrafenib onto the astrocyte backpack.

Experimental design and methods

Sub aim 1.1: Conjugate dabrafenib with MMP-2-degradable polypeptide and DBCO.

The C-terminus of the peptide will be conjugated to the free amine of dabrafenib (ChemieTek) via HATU coupling (Figure 1A). MMP-2 degradable peptide will be purchased (Biorbyt Ltd.) and 1 gram (1.2 mmol carboxylic acids) will be dissolved with 0.3 mmol 4-methyl morpholine in 5 mL anhydrous dimethyl formamide within a 25 mL round bottom flask. In a second 25 mL round bottom flask, 0.3 mmol 4-methyl morpholine, 4.8 mmol HATU (Chem-Impex) and 12 mmol dabrafenib (excess for favored kinetics) will be dissolved in 5 mL anhydrous dimethyl formamide. After mixing for 15 minutes, the contents of the second round bottom flask will be added to the first via cannula, for a final molar ratio of 1:0.5:4:10 (peptide:4 methyl morpholine:HATU:dabrafenib). The reaction will be stirred continuously for 24 hours, subsequently quenched, and purified by HPLC using a gradient of 20% to 90% acetonitrile in water. After purification, the aqueous peptide solution will be dried via lyophilization, resulting in a pure powder. Effective functionalization of dabrafenib will be quantified by HNMR and MALDI.

To enable conjugation of the peptide to astrocyte bearing pendant azides, the N-terminus of the peptide will be conjugated to the carboxylic acid of dibenzylcyclooctyne PEG4 acid (DBCO acid, BroadPharm) via DMTMM coupling (Figure 1B). 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), DBCO acid, and peptide precursor will be dissolved at a 1:10:1 molar ratio, in 10 mL deionized water within a 25 mL round bottom flask. The reaction will be stirred for 24 hours, subsequently quenched, and purified by HPLC using a gradient of 20 % to 90 % acetonitrile in water. After purification, the aqueous peptide solution will be dried via lyophilization, resulting in a pure powder. Effective functionalization of DBCO will be quantified by HNMR, as well as MALDI.

After tethering, drug activity will be assessed through in vitro assays using two MBM cell lines with the V600E BRAF mutation: WM4237 and M331[29], which will be gifted by Dr. Meenhard Herlyn at the Wistar Institute. The efficacy of DBCO-peptide-dabrafenib on targeting metastatic melanoma cells will be evaluated through proliferation and viability assays, which include cell staining with trypan blue and MTT assay, as well as assays for adhesion, migration, and invasion[29] through assays including cell adhesion assay to fibronectin bovine plasma coated plates, scratch assay, and 3D spheroid invasion assay.

Sub aim 1.2: Conjugate patient-derived astrocytes with dabrafenib-loaded MMP-2-degradable polypeptide.

iPSC-derived astrocytes will be metabolically labeled with pendant azides. Patient-derived stromal cells will be first differentiated to astrocytes as previously reported[30] and the astrocytes will be labeled with pendant azides as previously reported[31]. Then, the DBCO-peptide-dabrafenib will be assembled to the labeled astrocytes via click chemistry between DBCO and pendant azides. Specifically, astrocyte cell culture media will be conditioned with the prepared DBCO-peptide-dabrafenib. The terminal DBCO group will conjugate to astrocyte pendant azides via strain promoted azide alkyne addition.

Upon successful loading of the astrocyte backpack, its efficacy will be assessed through in vitro assays using WM4237 and M331 cells. Each cell line will be divided into three groups for different treatments: dabrafenib only, DBCO-peptide-dabrafenib, and astrocyte-peptide-dabrafenib. IC50 will be used as the measurement for this assay. Furthermore, the mobility of the astrocyte backpacks will be evaluated by culturing plain astrocytes and astrocyte-peptide-dabrafenib with WM4237 cell-conditioned media. Changes in migratory speed and direction will be tracked to confirm the ability of the astrocyte backpacks to target and migrate towards melanoma cells.

The release rate and total cumulative release of dabrafenib from the polypeptide-astrocyte carrier will be investigated in vitro utilizing a fluorogenic assay in combination with treatments of varying concentrations of collagenase-D, which will be used to synthetically mimic the biologically relevant concentrations of MMP-2 in the TME niche. The bioactivity of immobilized versus released dabrafenib will be assessed by direct comparison in a PrestoBlue assay using multiple melanoma cell lines. Released dabrafenib and cleaved peptide can be purified by HPLC for further studies. The bioactivity of released dabrafenib versus unmodified dabrafenib will be investigated in a similar approach, by matching conditional concentrations in serial dilutions.

Expected outcomes, potential pitfalls, and alternative approaches

Dabrafenib's drug profile and its interaction with the ATP-binding pocket of BRAF[32] are not expected to be affected by the conjugation to the MMP-2-degradable peptide and subsequent loading onto astrocytes.

However, in consideration of potential polypeptide remnants on dabrafenib post-cleavage and the potential impact of the conjugation, a liposome containing native dabrafenib can be used for conjugation with the polypeptide. Moreover, the exploration of other BRAFi for similar potential bioactivity will be conducted.

Additionally, activated astrocytes in proximity to melanoma cells may secrete MMP-2[22], which could potentially prematurely degrade the peptide chain before the astrocytes migrate into the tumor. This concern is less significant when the astrocyte remains in an inactive state. However, if non-specific, self-releasing of the drug from astrocytes is observed once melanoma cells enter the brain, a CRISPR-Cas9 procedure will be conducted to knockout the MMP-2 gene in astrocytes, preventing MMP-2 secretion and thus, the premature peptide chain digestion. If the issue persists, a comprehensive screening of proteinases secreted by astrocytes and melanoma cells will be performed, and the peptide linker will be adjusted to ensure that only the proteinases uniquely secreted by melanoma can degrade the peptide chain.

Specific aim 2: Encapsulate engineered astrocytes in TME-responsive microgel for in vivo delivery

Rationale

Given the different pH in the basic cerebrospinal fluid and the acidic TME[14], an acid-liable microgel will be designed to protect the astrocyte backpacks loaded with dabrafenib against environmental variations. Polyethylene glycol (PEG) serves as the structural backbone for encapsulating astrocyte backpacks due to its stability and reduced cell endosomal absorption, which has been widely applied in prolonging the drug clearance for treating brain tumors[33]. Additionally, PEG's ability to penetrate the BBB[34] is advantageous for the eventual clearance of the microgel, preventing prolonged residual presence. Lastly, PEG has been shown to promote little foreign body response, which will reduce the risk of adverse stimuli to the TME niche.

To make microgel responsive to the decreasing pH as it approaches the TME, PEG will be crosslinked through imines, created by aldehyde groups from photoactivated PEG-nitrobenzyl (PEG-oNb) and the amines from PEG-NH_2. The photoactivation step enhances spatiotemporal control of microgel crosslinking, ensuring homogeneous gelation and effective yet facile encapsulation of astrocytes. Moreover, 4 arm 20 kDA PEG was chosen because it is highly water soluble, easy to functionalize, and presents little isotropic swelling upon gelation. The percent modification of PEG, as well as the total polymer weight percent, will be investigated to optimize astrocyte viability and tune degradability for both long and short timescale release. Acidification of the TME niche progresses with increasing disease severity, which will in turn increase the rate of hydrogel degradation and subsequent astrocyte release. After the astrocyte backpacks are successfully encapsulated into the microgel, they will be injected into mouse models in vivo with either melanoma brain micro- or macrometastases to evaluate its efficacy of preventing and treating MBM in early and later stages.

Experimental design and methods

Sub aim 2.1: Encapsulate astrocyte backpack into acid-liable microgel.

Polyethylene glycol amine (4 arm 20 kDa PEG-NH_2, Jenkem) will be functionalized such that 2 of 4 arms are converted to nitrobenzyl groups, permitting network formation upon exposure to 405 nm light, correspondingly forming imine crosslinks (Figure 2A). 1 gram of 4 arm 20 kDa PEG-NH_2 (0.8 mmol amine) will be dissolved with 0.2 mmol 4-methyl morpholine in 5 mL anhydrous dimethyl formamide within a 25 mL round bottom flask. In a second 25 mL round bottom flask, 0.2 mmol 4-methyl morpholine, 3.2 mmol HATU, and 0.4 mmol hydroxyethyl photolinker (Nitrobenzyl, Advanced Chemtech) will be dissolved in 5 mL anhydrous dimethyl formamide. After mixing for 15 minutes to allow activation of carboxylic acids, the contents of the second round bottom flask will be added to the first via cannula, for a final molar ratio of 1:0.5:4:0.5 (amine:4 methyl morpholine:HATU:nitrobenzyl). The reaction will be stirred continuously for 24 hours, subsequently quenched, and then precipitated twice into excess (~20 fold by volume) ice cold diethyl ether to remove residual organic-soluble reagents. The precipitate will result in a yellowish-white powder, indicating successful nitrobenzyl conjugation. After drying overnight, the precipitate will be dissolved in deionized water and dialyzed for 3 days in an 8kDa regenerated cellulose membrane. The dialyzed solution will be lyophilized, and a yellowish white powder will be collected. Effective functionalization of the terminal amines and nitrobenzyls will be quantified by HNMR.

Rheological characterization of hydrogel formation will be investigated via photopolymerization by mixing PEG-oNB with phosphate buffered saline (PBS) and irradiating with 405 nm (Figure 2B). In situ photopolymerization of hydrogels will be investigated by varying light intensity and weight percent of polymer to achieve optimal mechanics for astrocyte culture, and degradation kinetics for subsequent release. Degradation

kinetics of hydrogels will be investigated by assessing multi-timepoint fold change in moduli after incubation of hydrogels in a variety of acidic pH buffers.

Viability of astrocyte “cellular backpack” encapsulation in bulk photoactivatable PEG imine hydrogels will be investigated and quantified with a calcein/ethidium homodimer assay via fluorescent microscopy. If cellular viability is comparable across hydrogels of similar mechanics, then astrocyte proteomic profile will be assessed to determine optimal conditions via multiplexed ELISA and/or protein set enrichment analysis. After optimizing cellular viability by tuning hydrogel mechanics (polymer weight percent) and network connectivity (functionalization), microfluidic encapsulation of cells within microgels will be investigated, and a microfluidic droplet generator will be purchased from a commercial supplier (uFluidix). Encapsulation efficiency, size distribution of microgels, and viability of microgel encapsulated cells will also be investigated and quantified with a calcein/ethidium homodimer assay via fluorescent microscopy. Lastly, clinically relevant injection forces will be investigated to determine if the astrocytes are sufficiently protected from injection shear and extensional flow forces.

Sub aim 2.2: Evaluate the efficiency of astrocyte backpack in early undetectable MBM and post-surgery tumor removal in vivo.

An autochthonous mouse model of MBM will be generated via mating Dct::TVA;$Braf^{V600E}$; $Cdkn2a^{lox/lox}$; $Pten^{lox/lox}$ + RCAS-Cre and RCAS-myrAKT1 gifted by Dr. Sheri L. Holmen from the University of Utah Health Sciences Center[35]. This model will develop spontaneous primary melanoma with a 79% incidence rate of brain metastasis in 6 weeks[36]. The development of MBM in individual mice will be confirmed through small-animal positron emission tomography (PET) and computed tomography (CT)[37]. Both sexes will be included, and all the mice will be 10-week-old for in vivo testing. All animal studies will be conducted in compliance with the guidelines established by the Duke Animal Care and Use Committee.

The current astrocyte backpack carries a single dabrafenib molecule. The corresponding intracranial dose of astrocyte backpacks will be calculated based on the effective dose from the previous in vitro study as a starting point (1.0 ×) and it will be escalated 2 levels further following a modified-Fibonacci dose-escalation scheme (2.0 × and 3.3 ×) for a total of three dose levels. All the control groups will be injected with a microgel encapsulating the native astrocytes labeled with azides for tracking.

To investigate the preventive and surveillance effects of astrocyte backpacks against the early stage micrometastasis, mice that have developed primary melanoma but do not yet exhibit detectable MBM through PET and CT imaging will be divided into four groups, each containing five mice. One group will be the control group, while the other three groups will receive gels with varying levels of astrocyte backpacks. Monitoring for MBM development will start from week 5.

To assess the effects of astrocyte-backpack-loaded microgels in assisting recovery from the surgical removal of MBM tumors and the prevention of relapses, mouse models will develop MBM tumors confirmed by PET and CT imaging and subsequently undergo surgical tumor removal. Following the surgery, both the control microgel and microgels encapsulating astrocyte backpacks at various levels, with excess astrocyte azides labeled by fluorophores, will be injected into the brain cavity. Over the next 12 weeks, fluorescence signals will be monitored to track the release of astrocyte backpacks into the cranial space, their migration to the TME, and their clearance from the brain. Subsequently, brain tissues will be collected for histological studies to investigate the impact of various levels of astrocyte backpacks on cavity healing, recruitment of immune cells, and prevention of secondary metastasis relapses.

Expected outcomes, potential pitfalls, and alternative approaches

PEG encapsulation is expected to provide protection for the astrocyte backpack and facilitate its release upon cues within the TME. While the synthesis of PEG-oNB has been previously documented, achieving target functionalization may be impeded by reaction efficiency. Thus, the stoichiometry of the reaction (nitrobenzyl concentration) will be assessed for target functionalization (2 out of 4 arms).

In case of a potential loss of stimulated migration capacity in iPSC-derived astrocytes, T cells have been proposed as an alternative "cellular backpack" for carrying dabrafenib.

The degradation times may significantly differ between in vitro and in vivo contexts. Consequently, gel formulation can be adjusted as needed, considering factors such as polymer weight percentage, the number of crosslinks, and the crosslinking mechanism.

For more confounded patient background where the generic MBM mouse model might not be representative, a library of patient-derived xenografts will be developed to generate a patient-derived MBM mouse model[38] for a more effective simulation.

Scientific rigor and statistical analysis

Data analysis and visualization will be conducted in R and Prism. Error bars will be presented as mean ± 95% standard error for the continuous measurements and median ± 95% standard deviation for the clinical scores. The statistical difference in means between groups will be evaluated using ANOVA with post-hoc Bonferroni test unless specified otherwise. A P-value smaller than 0.05 will be considered statistically significant.

Future directions

Upon the successful development of gel-protected astrocyte backpacks demonstrating the expected functionality and superiority over orally administered dabrafenib for MBM treatment, further investigations will be pursued. The primary focus will be the optimization of the ideal number of loaded dabrafenib molecules per astrocyte to enhance efficacy while maintaining sufficient cellular mobility. Additionally, the performance of dabrafenib-loaded astrocyte backpacks will be compared to other variants carrying single-agent BRAFi and the combination of dabrafenib with trametinib, a widely used MEK inhibitor in metastatic melanoma treatment, to assess potential synergistic effects. Ultimately, the exploration of 2-hydroxypropyltrimethyl ammonium chloride chitosan, a more soluble derivative of chitosan, as a substitute for PEG will be undertaken due to its anti-tumor properties. Further pharmacokinetic-pharmacodynamic (PKPD) studies of the astrocyte backpack loaded with dabrafenib will also be conducted to determine the optimal intracranial injection dosage for in vivo before the phase I clinical trial.

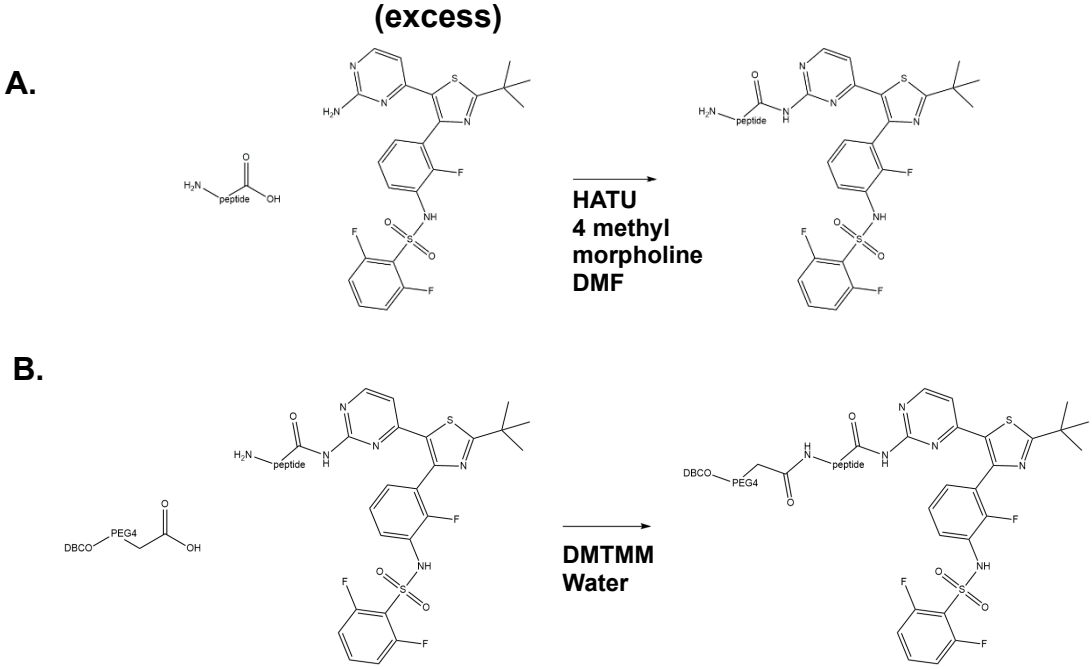


Figure 1. Schematic of synthetic approach to preparing DBCO-peptide-dabrafenib. Dabrafenib is introduced in large excess via HATU coupling to drive kinetic selectivity over homopolymerization (~10 fold molar excess). The peptide-dabrafenib precursor is then functionalized with DBCO-PEG4 acid via DMTMM coupling.

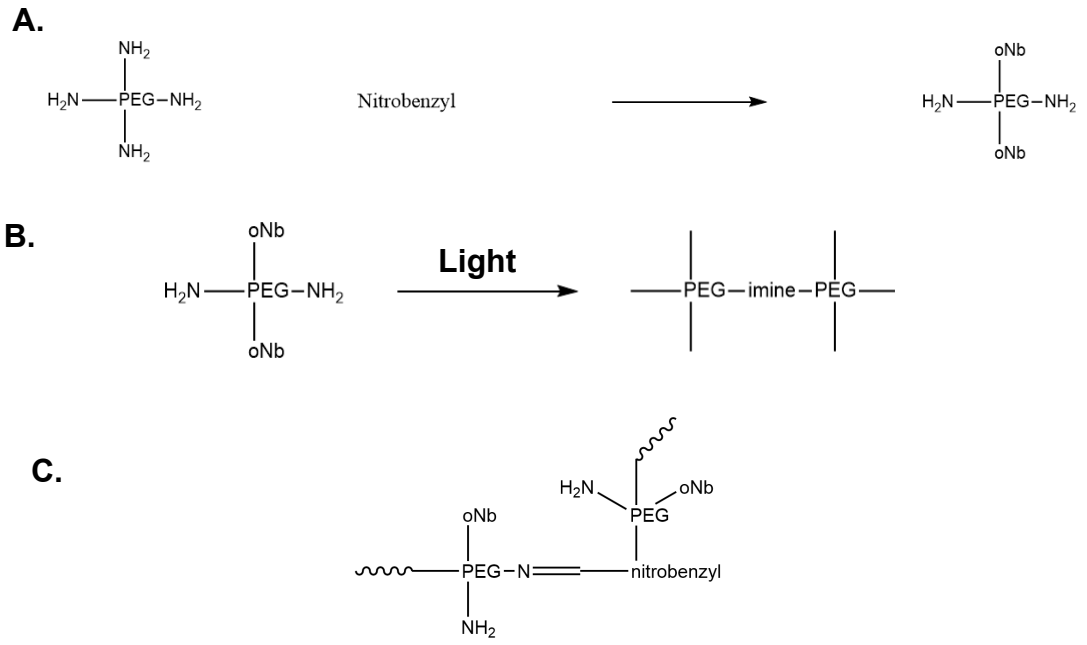


Figure 2. Schematic of synthetic approach to preparing multifunctional PEG decorated with amines and nitrobenzyls via HATU coupling. General crosslinking approach of multifunctional PEG with 405nm light to form network crosslinks.

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