Efficient brain targeting and therapeutic intracranial activity of bortezomib through intranasal co-delivery with NEO100 in rodent glioblastoma models

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OBJECTIVE Many pharmaceutical agents are highly potent but are unable to exert therapeutic activity against disorders of the central nervous system (CNS), because the blood-brain barrier (BBB) impedes their brain entry. One such agent is bortezomib (BZM), a proteasome inhibitor that is approved for the treatment of multiple myeloma. Preclinical studies established that BZM can be effective against glioblastoma (GBM), but only when the drug is delivered via catheter directly into the brain lesion, not after intravenous systemic delivery. The authors therefore explored alternative options of BZM delivery to the brain that would avoid invasive procedures and minimize systemic exposure.

METHODS Using mouse and rat GBM models, the authors applied intranasal drug delivery, where they co-administered BZM together with NEO100, a highly purified, GMP-manufactured version of perillyl alcohol that is used in clinical trials for intranasal therapy of GBM patients.

RESULTS The authors found that intranasal delivery of BZM combined with NEO100 significantly prolonged survival of tumor-bearing animals over those that received vehicle alone and also over those that received BZM alone or NEO100 alone. Moreover, BZM concentrations in the brain were higher after intranasal co-delivery with NEO100 as compared to delivery in the absence of NEO100.

CONCLUSIONS This study demonstrates that intranasal delivery with a NEO100-based formulation enables noninvasive, therapeutically effective brain delivery of a pharmaceutical agent that otherwise does not efficiently cross the BBB.

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Intranasal application of pharmacological agents has recognized therapeutic potential and avoids many of the above-described challenges to brain-targeted drug delivery. Its possible advantages include rapid nose-to-brain transport that avoids the BBB, simple application without invasive procedures, minimized systemic exposure, and less drug inactivation by the liver.\textsuperscript{11,12} While the exact mechanisms underlying intranasal drug delivery to the brain are not entirely understood, an accumulating body of evidence demonstrates that pathways involving cranial nerves (olfactory, trigeminal) connecting the nasal passages to the brain and spinal cord are important. In addition, pathways involving the vasculature, cerebrospinal fluid, and lymphatic system (glymphatics) have been hypothesized to be responsible, although one pathway may predominate, depending on the physicochemical properties of the therapeutic agent.\textsuperscript{4,11,12}

In the context of intracranial chemotherapy, studies of methotrexate, raltitrexed, and 5-fluorouracil in rats demonstrated that these antineoplastic agents could be directly transported into the brain via the olfactory pathway, resulting in significantly greater brain exposure than intravenous dosing.\textsuperscript{35,39,40} However, these studies did not investigate any antitumor efficacy, and in general there is a paucity of published reports on intranasal delivery as a means of cancer chemotherapy.\textsuperscript{34} A remarkable exception to this are reports of clinical phase I and II studies in Brazil, where perillyl alcohol (POH), a naturally occurring monoterpene, was successfully administered intranasally to patients with malignant glioma.\textsuperscript{8–10} In the United States, a standardized, highly purified, GMP-quality version of POH, called NEO100,\textsuperscript{5} currently is being evaluated in ongoing phase I/II studies that employ its intranasal application to patients with recurrent glioblastoma (NCT02704858).

Based on the lipophilic and other physiochemical properties of POH/NEO100,\textsuperscript{5} we hypothesized that this compound might also be suitable as an intranasal delivery vehicle for brain-targeted co-transport of chemotherapeutic agents that otherwise do not cross the BBB effectively. To establish proof of principle of this concept, we decided to use bortezomib (BZM), a proteasome inhibitor that is in clinical use for multiple myeloma.\textsuperscript{1} BZM has been demonstrated to have in vitro activity against glioma cells at nanomolar concentrations.\textsuperscript{3,21,37,42} Its poor penetration of the BBB is supported by a number of observations. For example, the brain-to-blood ratio of BZM was found to be 0.02 after administration of an intravenous bolus to rats.\textsuperscript{19} In studies with orthotopic mouse brain tumor models, there was no therapeutic efficacy of BZM when it was administered by a clinically relevant, i.e., intravenous, mode of application,\textsuperscript{22,44} consistent with results from clinical trials with recurrent malignant glioma patients, where intravenous BZM did not reveal clinical activity.\textsuperscript{15,25,29} In stark contrast, BZM did exert significant therapeutic activity when delivered by means of convection-enhanced delivery directly into the brain tumor.\textsuperscript{41}

In the current study with brain tumor–bearing mice and rats, we investigated whether intranasal delivery of BZM in combination with NEO100 would be able to result in significant levels of these agents in brain and tumor tissues and whether there was ensuing therapeutic activity.

Methods

Pharmaceutical Agents

Bortezomib was obtained from the pharmacy at the University of Southern California (USC)/Norris Comprehensive Cancer Center and was dissolved in saline solution at 2.5 mg/ml. NEO100 was produced in compliance with current good manufacturing practice (cGMP) regulations by Norac Pharma and was provided by NeOnc Technologies. It represents a highly purified (\(\geq 99\%\)) form of the natural monoterpene perillyl alcohol and is FDA approved for human clinical trials.

Cell Culture

Human U251 glioblastoma cells (purchased from Sigma-Aldrich), rat RG2 glioblastoma cells (American Tissue Culture Collection), and mouse GL26 glioma cells (kindly provided by Dr. Linda Liau, UCLA) were propagated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified incubator at 37°C and 5% CO\textsubscript{2} atmosphere. All cell culture reagents were provided by the Cell Culture Core Lab of the USC/Norris Comprehensive Cancer Center and prepared with raw materials from Cellgro/MediaTech; FBS was obtained from Omega Scientific.

MTT Assay

Methylthiazoletetrazolium (MTT) assays were performed as described earlier.\textsuperscript{6} Briefly, 5000 cells per well were seeded into 96-well plates. The next day, BZM (or solvent alone) was added, and 48 hours later the MTT assay was performed according to the manufacturer’s protocol (MilliporeSigma). Absorbance was measured using a microtiter plate reader (Molecular Devices) at 560 nm. Percent viability was calculated relative to vehicle-treated control cells. All experiments were performed at least in triplicate.

Intracranial Tumor Cell Implantation

All animal protocols were approved by the institutional animal care and use committee of USC, and all rules and regulations were followed during experimentation on animals. Animals were purchased from Harlan Inc. Human U251 cells (2 \(\times\) 10\textsuperscript{5} cells in 5 \(\mu\)l) were implanted into athymic, 6- to 8-week-old nude mice (immunodeficient xenograft model). As immunocompetent syngeneic models, we used 6- to 8-week-old C57BL/6 mice implanted with mouse GL26 cells and 4- to 6-week-old Fisher 344 rats implanted with rat RG2 cells (0.5–1 \(\times\) 10\textsuperscript{4} cells in 5 \(\mu\)l). In all cases, tumor cells were placed orthotopically (into the frontal lobe) as described earlier.\textsuperscript{6} Buprenorphine-SR was used for postoperative analgesia.

Intranasal Drug Delivery

Intranasal administration of drugs was performed with 3/4-inch-long 24-gauge plastic SurFlash intravenous cathe-
years (Terumo Corp.) attached to a 1-ml syringe. For mice, a total volume of 20 μl was slowly applied to alternating nostrils over the course of 5 minutes; for rats, a volume of 50 μl was given over 20 minutes. For pharmacokinetic measurements, a single dose was applied. For determination of therapeutic impact (survival studies), intranasal treatment was administered once daily for 14 consecutive days, followed by 7 days without treatment. After these 3 weeks, the treatment cycle was repeated. For comparison purposes, BZM was also given via intravenous injection. In this case, we followed the standard clinical schedule of twice-weekly injections (tail vein) over the course of 2 weeks, followed by a 10-day rest period. In all therapeutic studies, the overall dose of BZM was 1 mg/kg for each cycle.

Collection of CSF From the Experimental Animals

Upon euthanasia (ketamine plus xylazine overdose, followed by thoracotomy), the rat’s head was fixed in a stereotaxic instrument in the horizontal plane, with the rest of the body lying vertically, forming a 90° angle between the body and the head. A 26-gauge needle was inserted through the reservoir between the occipital protuberance and the spine of the atlas. Approximately 100 μl CSF was withdrawn from each animal. Three independent samples were collected from different time points and analyzed for BZM content by high-performance liquid chromatography (HPLC).

Serum Analysis

At the completion of the experiment, animals were euthanized (as above) and at least 100 μl blood was collected into K₂EDTA-coated BD Vacutainer tubes (Becton Dickinson). Tubes were gently inverted 8–10 times, followed by centrifugation at 2000 rpm for 3 minutes. The plasma was then frozen at –80°C and sent to MicroConstants Inc. for analysis.

Brain Tissue Analysis

Immediately following euthanasia, rat brains were collected, separated into 5 anatomical regions, and weighed. These regions (R) contained the following structures: R1—cerebrum, putamen, lateral ventricles, olfactory bulb (tract); R2—thalamus, hypothalamus, third ventricle, optical nerves; R3—midbrain and mesencephalic aqueduct; R4—pons, fourth ventricle, and cerebellum; and R5—medulla oblongata and spinal cord.

Bioanalysis of NEO100 and Its Perillic Acid Metabolite

For NEO100 analysis, rat plasma (in K₂EDTA anticoagulant) and brain homogenate samples were extracted in dichloromethane; 4-isopropylbenzyl alcohol served as the internal standard (IS). The drug and IS were then oxidized into their respective aldehydes and subsequently derivatized. Perillaldehyde, if present, was not distinguished from perillyl alcohol. The extracts were analyzed by HPLC using an ACE 5 C18-AR column (Macmod). The mobile phase was nebulized using heated nitrogen in a Z-spray source set to positive ionization mode. The ionized compounds were detected using tandem mass spectrometry (MS/MS).

For PA analysis, rat plasma and brain homogenate samples were precipitated with acetonitrile and derivatized into an amide; 4-isopropylbenzoic acid was the IS. The extracts were analyzed by HPLC using an Allure Biphenyl column (Restek). The mobile phase was nebulized using heated nitrogen in a Z-spray source set to positive ionization mode. The ionized compounds were detected using MS/MS.

Pharmacokinetic Analysis of BZM

Brain tumor tissues and contralateral normal brain tissues from BZM-treated rats were homogenized according to standard protocols for HPLC analysis. In brief, the tissues were homogenized with 10 mM ammonium acetate and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatants were collected and stored at –80°C for subsequent analysis. Samples were passed through a 3-kDa molecular weight cutoff centrifuge filter prior to analysis. HPLC was performed on a Shimadzu i-Series Plus system (Shimadzu) with analysis carried out using a Restek Roc C18 column with a linear gradient of 5%–95% acetonitrile over 15 minutes. BZM was detected using a diode array detector focusing on a wavelength of 230 nm.

HPLC Analysis of BZM in CSF and Serum

Samples from CSF and serum were prepared for analysis by centrifugation at 14,000g for 20 minutes, followed by 4-kDa molecular weight filtration through a nylon filtration unit. The filtrate was placed in auto-sampler vial instrumentation: The mobile phase was a mixture of mobile phase A (water-acetonitrile–trichloroacetic acid, 95:5:0.1, v/v/v) and phase B (water-acetonitrile–trichloroacetic acid, 5:95:0.1, v/v/v). A gradient elution was applied with the following program: 2 minutes with 95%A:5%B; 10-minute linear gradient to 95%B at a flow rate of 1.0 ml/min. Detection was monitored at a wavelength of 268 nm. The injection volume was 10 μl.

Stability Testing of BZM in NEO100

BZM was placed into a solution of 0.3% NEO100. An aliquot was immediately removed and placed in a sample vial. The remaining solution was placed in a 37°C incubator with aliquots being removed at different time points up to 24 hours. The samples were analyzed for the presence of BZM using the described analysis method, with 3 runs for each sample; 100% BZM was set at the mean of 3 time zero measurements.

Statistical Analysis

Animal survival data were plotted using the Kaplan-Meier method. One-way ANOVA was used for the overall test for differences. Pairwise comparisons were performed using the Tukey method of adjusting for multiple comparisons. Log-rank (Mantel-Cox) test was applied for the comparison of survival curves. A statistical evaluation result of p < 0.05 was considered significant.

Results

BZM is known to exert cytotoxic activity in multiple myeloma and a few other tumor cell lines in the low mi-
We investigated its in vitro potency against established glioma cell lines derived from mouse (GL26), rat (RG2), and human (U251). We established that BZM was able to kill these tumor cells at IC$_{50}$ values of 20 nM, 18 nM, and 13 nM, respectively (Fig. 1), indicating that the observed high BZM sensitivity of these cells would be suitable for our subsequent in vivo investigations.

Because our objective was to characterize NEO100-mediated intranasal drug delivery as an effective means to target BBB-impermeable drugs to the brain, we first determined the concentration of NEO100 in different parts of the brain. NEO100 was administered into the nostrils of rats and their brains were collected and dissected at different time points thereafter. Tissue concentrations of POH, as well as its main metabolic product, perillic acid, were determined by mass spectrometry. As shown in Fig. 2A, POH was readily and rapidly detectable in all sections of the brain within 5 minutes after completion of NEO100 delivery. Its presence was short-lived, with a rapid drop in its tissue concentrations at 15 minutes, and complete dis...
appearance at 60 minutes after drug delivery, consistent with POH’s known very short in vivo half-life. In comparison, perillic acid was present for much longer. It could be detected at each time point investigated, i.e., at 5, 15, 30, and 60 minutes after completion of intranasal delivery of NEO100 (Fig. 2B). Altogether, these data provide the first direct evidence that POH is present in the brain after intranasal application.

Having established that POH is able to enter the brain after nasal delivery, we next investigated nasal co-delivery of BZM together with NEO100 in rats with syngeneic, orthotopically implanted glioma cells. Rats were implanted with RG2 cells into the left brain hemisphere, and after 3 weeks nasal drug delivery was performed. Animals received BZM alone or BZM formulated in 0.3% NEO100 into their nostrils, and tumor tissue and contralateral normal brain were collected at different time points thereafter, from 0 minutes (immediately after completion of nasal delivery) up to 60 minutes. For comparison purposes, we also determined brain entry of BZM after systemic (intravenous via tail vein) drug delivery to rats. Figure 3A shows that BZM concentrations in the brain were more than 100-fold lower than BZM concentrations in serum, confirming that BZM indeed crosses the BBB very poorly. In comparison, brain BZM levels were much higher after intranasal delivery: As shown in Fig. 3B, BZM was detectable both in tumor tissue and in normal brain at all time points analyzed. At time 0 (i.e., immediately after intranasal delivery), the amount of BZM in both tissues was about 3 times greater when BZM was co-delivered with NEO100, as compared to nasal BZM administration in the absence of NEO100. At subsequent time points (5, 15, 30, and 60 minutes after nasal delivery), co-delivery yielded higher BZM levels as well, although the difference was smaller (from 1.0-fold to 2.2-fold). These data indicate that intranasal delivery, particularly in combination with NEO100, can achieve much higher brain concentrations of BZM, as compared to intravenous delivery.

We next determined whether nasal co-delivery of BZM with NEO100 would achieve therapeutic outcomes in an orthotopic mouse xenograft model, where human U251 cells were implanted into the brains of athymic nude mice. These animals received nasal delivery of BZM, with or without NEO100 coformulation, or intravenous BZM, also either alone or together with NEO100. Control mice received vehicle alone. As shown in Fig. 4A, intravenous drug delivery did not result in therapeutic activity, i.e., it did not significantly extend survival of brain tumor–bearing animals over those that received vehicle alone. This was true for both modes of drug delivery, BZM alone and BZM combined with NEO100, and was consistent with reports by others22,41 that intravenous BZM is unable to exert significant therapeutic efficacy after systemic delivery. In contrast, when BZM was formulated with NEO100 and delivered through the nose, there was a significant extension of survival of treated animals over those that received vehicle alone, BZM alone, and NEO100 alone (Fig. 4B). The median survival of these groups of mice was 23 (vehicle), 27 (NEO100), 31 (BZM), and 41 days (NEO100 + BZM). Compared to vehicle, the 4-day extension of survival by NEO100 was not statistically significant (p = 0.08), whereas the 8-day extension by BZM (p = 0.037) and 18-day extension by NEO100 + BZM (p = 0.002) represented significant differences. As well, in comparison to treatments with the individual components, the combination treatment of NEO100 + BZM was superior to BZM alone (p = 0.033) and NEO100 alone (p = 0.02). Combined, these results provide proof of principle that the lack of therapeutic activity of BZM against brain-localized tumors can be overcome if BZM is formulated with NEO100 and administered via the nose.

We also performed a small-scale pharmacokinetic study to determine the extent of BZM penetration into the CNS. We measured the concentration of BZM in CSF and serum after intranasal delivery of BZM with or without...
Discussion

A large number of potent therapeutic agents are unable to unfold their benefits against disorders of the brain, because the BBB minimizes or completely blocks their brain access from the systemic circulation. While there are many therapies against peripheral diseases, there is a paucity of effective agents for central disorders. This detrimental situation is particularly evident in the case of malignant glioma, for which only one new chemotherapeutic agent, temozolomide (TMZ), has been added to clinical practice since the turn of the millennium. And even in this encouraging example, the BBB interferes with optimal outcomes: the ratio of drug concentrations between the cerebrospinal fluid (CSF) and patient plasma is 0.2, meaning that only a fraction of systemically available TMZ enters the brain.\(^\text{26,27}\) Not surprisingly, the ensuing therapeutic benefit is limited; in a landmark phase III clinical trial with selected, well-performing patients, the addition of TMZ extended median survival by only 2.5 months, from 12.1 to 14.6 months.\(^\text{36}\) While this relatively small improvement was generally considered as a major breakthrough, it also reflects the low bar of expectations and underscores the urgent need for greater advancements in this field.

In the current preclinical study, we provide evidence that nasal delivery can achieve chemotherapeutic drug concentrations in the brain that are sufficiently high to exert significant activity of a drug that otherwise crosses the BBB only poorly. We chose BZM as a model compound because of indications that it might exert activity against glioblastoma (GBM), if only it could gain greater access to the intracranial tumor site. For instance, prior reports had detailed BZM’s ability to kill GBM cells in vitro,\(^\text{3,21,37,42}\) and co-transport might be facilitated by the amphipathic nature of POH and the close proximity of the 2 components. Although the veracity of either of these 2 mechanisms awaits validation, the fact remains that we have documented nasal delivery resulted in significant therapeutic benefit of BZM for animals with orthotopic GBM (Fig. 4B). While nasal delivery of BZM alone yielded promising activity in itself, the greatest effect was achieved when it was formulated with 0.3% NEO100. The underlying principle of this enhancement is currently under investigation, and there are at least 2 conceivable scenarios. First, it is possible that NEO100 acts as a vehicle that enhances the therapeutic impact against intracranial GBM when delivered systemically, i.e., via intravenous delivery,\(^\text{46}\) which is also observed in our mouse model (Fig. 4A).

In stark contrast, we find that switching to intranasal delivery resulted in significant therapeutic benefit of BZM for animals with orthotopic GBM (Fig. 4B). While nasal delivery of TMZ alone yielded promising activity in itself, the greatest effect was achieved when it was formulated with 0.3% NEO100. The underlying principle of this enhancement is currently under investigation, and there are at least 2 conceivable scenarios. First, it is possible that mixing BZM with NEO100 created a combination effect, where both agents contributed to the killing of tumor cells. This model would be consistent with a large number of reports that demonstrated anticancer activity of POH in a variety of experimental conditions and the clinical setting (see detailed references in the 2015 article by Chen et al.\(^\text{41}\)).

Lastly, we addressed the question whether NEO100 would affect the stability of BZM, and this way perhaps affect the differential readouts and therapeutic outcomes described above. BZM was incubated at 37°C in the presence or absence of NEO100 in vitro, and BZM concentrations were measured at times 0, 4, and 24 hours. As shown in Fig. 5B, BZM remained stable during this incubation period, i.e., BZM concentrations at all 3 time points were fairly similar, indicating that the presence of NEO100 did not affect BZM stability or turnover.
significantly increased when the drug was co-delivered with NEO100.

The limitations of our study include the model system used. As is well known,17,18 the anatomy of the nasal cavity of rodents—in particular, the relative surface area of the olfactory epithelium and vascularization of the nasal lining—is quite different from that of humans. It therefore is not entirely clear how well this co-delivery approach can be translated from our rodent studies to the clinic. However, an optimistic view will consider that the general concept of intranasal, brain-targeted delivery is supported by encouraging results obtained in clinical trials with other agents. For example, intranasal insulin has resulted in measurable beneficial effects in patients with Alzheimer’s disease.19 A clinical study with healthy volunteers provided support for direct nose-to-brain transport of oxytocin, independent of blood absorption.20 And, as detailed further above, perillyl alcohol on its own has revealed brain-targeted activity after intranasal delivery to patients with recurrent GBM.21-23 It therefore does not seem unreasonable to opine that the principle of NEO100-mediated co-delivery could be translatable to humans.

In general, a variety of direct and indirect transport routes are potentially available to carry intranasal BZM to the brain.11,12 For instance, BBB-permeable agents that are administered intranasally by nasal sprays may enter the systemic circulation via uptake by the vasculature of the nasal lining or the lung epithelium, or to a lesser extent through postnasal drip into the stomach and transmission to the portal vein. Once present in the general circulation, traversing the BBB will constitute the predominant mechanism of brain entry for such agents. In contrast, in the case of BZM/NEO100 intranasal co-delivery, direct nose-to-brain transport appears to represent the primary mechanism of drug brain entry. This conclusion is based on our following observations: 1) Intravenous delivery of BZM, with or without NEO100, does not result in therapeutic impact on brain-localized tumors (Fig. 4A), indicating lack of sufficient brain entry from the systemic circulation. This is consistent with a BZM brain/serum ratio of only 1% (Fig. 3A). 2) Intranasal co-delivery of BZM + POH exerts significant brain-targeted anticancer activity (Fig. 4B). Importantly, under these treatment conditions, the presence of NEO100 creates a 10-fold increase in BZM concentrations in the CSF, but not in the serum (i.e., BZM concentrations in the serum are the same, irrespective of the presence of NEO100) (Fig. 5A). This latter observation argues against a model in which serum levels of BZM are decisive (despite their significant amounts), but instead poses direct nose-to-brain transport as the critical component of POH-mediated brain entry of BZM. While we did not investigate further details of this latter route, it is likely that neuronal transport mechanisms (olfactory, trigeminal24) might be involved.

Conclusions

In summary, we are providing proof of principle that a pharmacological agent with poor BBB penetration can effectively be delivered to the brain via intranasal delivery, and that this effect can be further optimized by using NEO100 as a component of the formulation. It will be important to determine whether this noninvasive principle of nose-to-brain transport can be expanded to enable the NEO100-based delivery of other brain-targeted agents with poor BBB penetration. Further validation of this new approach will set the stage for broader applications beyond cancer, potentially addressing other disorders of the brain, such as neurological diseases or drug abuse conditions.

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Disclosures
Dr. Chen reports an ownership interest (founder and stakeholder) in NeOnc Technologies, Los Angeles, CA.

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Conception and design: Chen, Hofman, Schönthal. Acquisition of data: Wang, Swenson, Cho, Hofman. Analysis and interpretation of data: Wang, Swenson, Cho. Drafting the article: Wang, Swenson, Cho, Schönthal. Critically revising the article: Chen, Swenson, Cho, Hofman, Schönthal. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Chen. Statistical analysis: Wang, Swenson. Administrative/technical/material support: Wang. Study supervision: Chen, Hofman, Schönthal.

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