Fluorescence Identification of Head and Neck Squamous Cell Carcinoma and High-Risk Oral Dysplasia With BLZ-100, a Chlorotoxin-Indocyanine Green Conjugate

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IMPORTANCE Surgical cure of head and neck squamous cell carcinoma (HNSCC) remains hampered by inadequately resected tumors and poor recognition of lesions with malignant potential. BLZ-100 is a chlorotoxin-based, tumor-targeting agent that has not yet been studied in HNSCC.

OBJECTIVE To evaluate BLZ-100 uptake in models of HNSCC and oral dysplasia.

DESIGN, SETTING, AND PARTICIPANTS This was an observational study (including sensitivity and specificity analysis) of BLZ-100 uptake in an orthotopic xenograft mouse model of HNSCC and a carcinogen-induced dysplasia model of hamster cheek pouches.

INTERVENTIONS Various HNSCC xenografts were established in the tongues of NOD-scid IL2Rgama null (NSG) mice. BLZ-100 was intravenously injected and fluorescence uptake was measured. To induce dysplasia, the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) was applied to the cheek pouch of Golden Syrian hamsters for 9 to 16 weeks. BLZ-100 was subcutaneously injected, and fluorescence uptake was measured.

MAIN OUTCOMES AND MEASURES The signal-to-background ratio (SBR) of BLZ-100 was measured in tumor xenografts. To calculate the sensitivity and specificity of BLZ-100 uptake, a digital grid was placed over tissue sections and correlative histologic sections to discretely measure fluorescence intensity and presence of tumor; a receiver operating characteristic (ROC) curve was then plotted. In the hamster dysplasia model, cheeks were graded according to dysplasia severity. The SBR of BLZ-100 was compared among dysplasia grades.

RESULTS In HNSCC xenografts, BLZ-100 demonstrated a mean (SD) SBR of 2.51 (0.47). The ROC curve demonstrated an area under the curve (AUC) of 0.89; an SBR of 2.50 corresponded to 92% sensitivity and 74% specificity. When this analysis was focused on the tumor and non-tumor interface, the AUC increased to 0.97; an SBR of 2.50 corresponded to 95% sensitivity and 91% specificity. DMBA treatment of hamster cheek pouches generated lesions representing all grades of dysplasia. The SBR of high-grade dysplasia was significantly greater than that of mild-to-moderate dysplasia (2.31 [0.71] vs 1.51 [0.34], \( P = .006 \)).

CONCLUSIONS AND RELEVANCE BLZ-100 is a sensitive and specific marker of HNSCC and can distinguish high-risk from low-risk dysplasia. BLZ-100 has the potential to serve as an intraoperative guide for tumor margin excision and identification of premalignant lesions.
P ositive margin status remains a poor prognostic indicator of local recurrence and long-term survival in head and neck squamous cell carcinoma (HNSCC).1 Positive margins are largely responsible for primary site recurrences and carry a 2-to-3-fold increased risk of all-cause mortality.2-5 Obtaining wider surgical margins may mitigate poor oncologic outcomes. However, in the head and neck, overaggressive resection is often detrimental to cosmesis and postoperative speech and swallow function.6 Minimally invasive surgical approaches, such as transoral laser microsurgery and transoral robotic surgery, attempt to achieve satisfactory oncologic outcomes while avoiding the morbidity of open surgery.7 With the inherent access limitations of these approaches, there is an increasing need to confidently identify the tumor and non-tumor interface.

Furthermore, the ability to identify lesions with increased risk for malignancy is crucial. Compared with mild-to-moderate dysplasia, severe dysplasia and carcinoma in situ (CIS) have a malignant transformation rate 2-fold greater in the oral cavity8 and 3-fold greater in the larynx.9 Clinically, the management of mild-to-moderate dysplasia can vary from observation and biopsy to definitive treatment. There is general consensus that severe dysplasia and CIS warrant aggressive treatment owing to their higher risk of malignant transformation. However, high-risk and low-risk dysplasia do not have unique phenotypical features, and given the lack of reliable clinical markers of dysplasia, the standard of care relies on repeated biopsies and histopathological staining.

Chlorotoxin (CTX)-based agents are peptide conjugates with natural tumor-binding qualities. Derived from the venom of the scorpion Leiurus quinquestriatus, CTX demonstrates unique binding properties in gliomas10,11 and a variety of other solid tumors.11,12 While our first CTX conjugate (known as “Tu-mor Paint”) used Cy5.5 as its fluorophore, the current conjugate, called BLZ-100, uses indocyanine green (ICG).13 The binding target of CTX conjugates has not yet been clearly defined but is thought to involve lipid rafts containing the membrane proteins annexin A214 and matrix metalloproteinase-2.15 BLZ-100 uptake in HNSCC has not been previously demonstrated, and to our knowledge, there has been no prior study of chlorotoxin conjugates in oral dysplasia. Using an orthotopic xenograft mouse model,16 we sought to establish BLZ-100 uptake in human HNSCC tumor xenografts and to determine the sensitivity and specificity of this uptake. Moreover, using a well-adopted model of oral dysplasia induced by the application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) in hamster cheek pouches,17-19 we tested the ability of BLZ-100 to discriminate among the stages of dysplasia and to highlight high-risk from low-risk dysplasia.

Methods

HNSCC Cell Lines
This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Fred Hutchinson Cancer Research Center and the Infectious Disease Research Institute in Seattle, WA. Three established human HNSCC lines were used. PCI-15B is derived from a metastatic lymph node of a primary oral cavity squamous cell carcinoma (SCC), and expresses green fluorescent protein (GFP).20 UM-SCC-22B is derived from a metastatic lymph node of a hypopharyngeal SCC,21,22 UM-SCC-47 is derived from a tongue SCC and is human papilloma virus (HPV) positive.23,24 Cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptavidin at 37°C in 5% carbon dioxide. As a fourth example of HNSCC, we included a low-passage, non-GFP-labeled primary culture, FHCRC/UW-SCC1. This primary culture was developed from a floor-of-mouth SCC and passage less than 20 times prior to xenograft injection (eMethods, and eFigure 1 in the Supplement). FHCRC/UW-SCC1 cells were maintained in serum-free keratinocyte media in identical conditions.

Imaging Agents
BLZ-100 was provided by Blaze Bioscience Inc. Briefly, an amine-reactive ester form of ICG (Sigma Aldrich) was conjugated to chlorotoxin using standard amine-reactive chemistry. The reaction proceeded in the dark at room temperature and was monitored by reversed-phase, high-performance liquid chromatography (HPLC). Indocyanide green- (ICG) free dye was removed by reversed-phase HPLC and fractions corresponding to the conjugate were pooled and lyophilized. The lyophilized conjugate was formulated in 10mM Tris, 5% mannitol (pH 7.2) to a final concentration of 2 mg/mL. For control injection experiments, ICG was reconstituted according to manufacturer’s instructions in sterile water and diluted in phosphate-buffered saline (PBS) to concentrations equimolar to BLZ-100.

Mouse Xenograft Model
NOD- scid IL2R gamma null (NSG, stock No. 005557, Jackson Laboratory) mice were housed and treated according to approved IACUC protocol at the Fred Hutchinson Cancer Research Center. Male and female mice at least 4 months of age were maintained on a 12-hour light cycle and fed an alfalfa-free diet.

In preparation for xenografting, cells were washed in PBS and resuspended in a 1:1 mixture with PBS and phenol-red-free Matrigel matrix (BD Biosciences) at a concentration reflective of their rapidity of migration in culture24: PCI-15B, 2.2×10⁴ cells/20 μL; UM-SCC-22B, 3.4×10⁴ cells/20 μL; UM-SCC-47, 3.1×10⁴ cells/20 μL; FHCRC/UW-SCC1, 7.5×10⁴ cells/20 μL. Tumor injectate (20 μL) was infiltrated into the antero-lateral tongue of NSG mice, as previously described.16 Based on prior study16 and additional time course and dose experiments in U87 glioma xenografts (eMethods, eFigure 2, and eTable 1 in the Supplement), tumor-bearing mice were injected with 6-nmol BLZ-100 via tail vein and were killed after 24 hours via carbon dioxide inhalation. To facilitate exposure of the oral cavity and cervical region, the anterior jaw and cervical skin were removed. Reflected light and fluorescent images were obtained using the Xenogen-IVIS Spectrum Imaging System (Caliper Life Sciences) using 465-nm excitation/520-nm emission for GFP and 745-nm excitation/820-nm emission for BLZ-100 and ICG imaging.
Tongues were harvested and divided at the tumor midline. One portion was fixed in 10% neutral-buffered formalin and paraffin-embedded for immunohistochemical analysis. The contralateral portion was frozen in optimal cutting temperature embedding compound, and 30-μm sections were cut for fluorescence imaging using the LI-COR Odyssey CLx scanner (LI-COR Inc) at the 800-nm channel with 21-μm resolution. Serial 12-μm sections were cut for standard hematoxylin-eosin staining.

Hamster Dysplasia Model
Golden Syrian hamsters (Charles River Laboratories) were housed at the Infectious Disease Research Institute, Seattle, Washington, and treated according to the approved IACUC protocol and US Department of Agriculture regulations. Male hamsters at least 3 months of age were maintained on 12-hour light cycles and fed a standard rodent diet.

As previously described, 17-19 0.9% DMBA (Sigma-Aldrich) dissolved in mineral oil was topically applied to the right mucosal cheek pouch 3 times a week under brief isoflurane anesthesia. Hamsters were treated with DMBA for 6, 9, or 12 to 16 weeks. A treatment-control cohort was treated with mineral oil only for the durations listed above. After treatment completion, hamsters were subcutaneously injected with 1 mg/kg of BLZ-100. An injection-control cohort was treated with DMBA for the durations listed herein and subsequently injected with 1mg/kg of ICG-free dye. Dosage and timing of imaging were selected based on prior unpublished time course experiments with subcutaneous injection in mouse flank tumor models.

Twenty-four hours following injection, hamsters were killed via carbon dioxide administration and thoracotomy. A subset of hamsters was imaged using the Xenogen-IVIS imaging system for qualitative purposes. Bilateral cheek pouches were resected en bloc and imaged with the Odyssey CLx scanner using the 800-nm channel with 21-μm resolution. Regions in the cheek pouch correlating to specific fluorescence measurements were carefully dissected, fixed in 10% neutral-buffered formalin, and paraffin embedded for hematoxylin-eosin staining. Representative images were captured with the Aperio ScanScope AT (Leica Biosystems) using the 20× objective.

Histologic Grading of Dysplasia
Based on previously published grading scales,25,26 a 5-point dysplasia scale was used. Hematoxylin-eosin–stained specimens were submitted to a board-certified veterinary pathologist (S.E.K.) who was blinded to treatment type, treatment duration, and fluorescence signal. Each specimen was graded based on at least 3 tissue sections sectioned 10-μm apart. Specimen grades were assigned according to the highest grade of dysplasia present.

Data Acquisition and Analysis
Fluorescence was measured in radiant efficiency using Living Image software, (version 4.0; PerkinElmer Inc) for Xenogen-IVIS images and in fluorescence counts using Image Studio software, (version 3.0; LI-COR, Inc) for Odyssey images. Statistical analysis was performed using PASW statistics software (version 18.0; IBM). t Test was used to quantitatively compare fluorescence, except where otherwise noted. The analysis of variance (ANOVA) test was used to compare fluorescence across several categorical groups (ie, cell line, dysplasia grade). Pearson correlation was used to analyze the relationship between GFP and BLZ-100 fluorescence. Statistical significance was defined as P < .05.

Results
BLZ-100 Uptake in HNSCC Xenografts
PCI-15B (n = 5), UM-SCC-22B (n = 3), and UM-SCC-47 (n = 6) xenografts were established onto the tongues of NSG mice. Tumor growth was evident after a mean (SD) incubation period of 28.2 (5.0) days for PCI-15B, 30.0 (0.0) days for UM-SCC-22B, and 21.0 (0.0) days for UM-SCC-47 xenografts. Tumor diameter was 3.0 (2.2) mm for PCI-15B, 4.8 (2.8) mm for UM-SCC-22B and 4.6 (1.7) mm for UM-SCC-47 tumors; these differences in size were not significant (P = .25).

Mice were imaged 24 hours after injection with BLZ-100. The signal-to-background ratio (SBR) was calculated by dividing the absolute fluorescence of tumor by the absolute fluorescence of surrounding normal tissue. A control cohort of PCI-15B xenografts injected with ICG-free dye (n = 3) demonstrated minimal fluorescence signal in regions correlating with tumor (mean [SD] signal-to-background-ratio [SBR] 0.97 [0.03]), (Figure 1A and B). Similarly, control mice without tumors that were injected with BLZ-100 (n = 5) demonstrated minimal fluorescence within the tongue (SBR, 1.08 [0.16]) (Figure 1C and D). In tumors expressing GFP (PCI-15B) (n = 5), the signal from BLZ-100 correlated highly with that from GFP in the primary tongue tumors (r = 0.92, P = .03) (Figure 1E and F). The SBRs of BLZ-100 in tumor xenografts were as follows: PCI-15B, 2.47 (0.36); UM-SCC-22B, 2.54 (0.39); and UM-SCC-47, 2.76 (0.43) (Figure 1G).

To investigate potential differences in BLZ-100 uptake between established tumor cell lines and a primary culture system, we xenografted 8 tumors from FHCRC/UW-SCC1, an early-passage HNSCC cell culture. FHCRC/UW-SCC1 tumors were grown for 20.6 (4.0) days and measured 3.8 (0.9) mm at the largest diameter. There was no significant difference in tumor size among all tumor types (P = .32). The SBR in FHCRC/UW-SCC1 tumors was 2.34 (0.56) (Figure 1G). There was no significant difference in SBR among tumor types (P = .43). The combined SBR for all tumor types was 2.51 (0.47). Compared with the SBR of ICG-free dye, the SBRs of BLZ-100 in PCI-15B, UM-SCC-22B, UM-SCC-47, and FHCRC/UW-SCC1 tumors were each significantly greater (P < .01 for all comparisons).

One mouse demonstrated a suspicious cervical mass, which was confirmed as metastatic SCC on histologic analysis (eFigure 3 in the Supplement). Using contralateral normal tissue as background, the SBR of BLZ-100 in this region was 2.26, while the SBR of GFP was 1.79 (Figure 1E and F).

Sensitivity and Specificity Analysis
To measure the in situ diagnostic performance of BLZ-100, we measured the sensitivity and specificity of BLZ-100 tumor uptake using a digital grid to map fluorescence intensity in tissue.
The SBR was obtained by dividing the fluorescence intensity of an individual box by the average intensity of a normal tissue region. A matching grid was overlaid on serial histologic sections to determine the presence of tumor. Grid boxes partially containing tumor were only classified as “positive” if tumor comprised more than 50% of the boxed area. Each box had a width and height of 0.3 mm.

Using the digital grid (Figure 2A), 843 measurements of fluorescence intensity and corresponding tumor status were made; 140 boxes were tumor-positive, and 703 were tumor-negative. The SBRs (SD) were 4.90 (1.86) in tumor-containing grid boxes and 2.06 (2.03) in non-tumor-containing grid boxes. A receiver operating characteristic (ROC) curve was calculated, which demonstrated an area under the curve (AUC) of 0.89 (Figure 2B), with an SBR of 2.5 corresponding to 92% sensitivity and 74% specificity.

BLZ-100 uptake was most intense at the periphery of the tumors (Figure 2A). Because the SBR at the tumor periphery is arguably more significant for complete tumor resection intraoperatively, we repeated our ROC analysis to
focus solely on boxes involving the true tumor margin. Using normal tissue lying 0.9 to 1.2 mm away from the tumor margin as background, the mean (SD) SBR was 5.26 (2.06) in tumor-containing grid boxes and 1.43 (1.01) in non–tumor-containing grid boxes. An ROC curve based on these data demonstrated an AUC of 0.97, and an SBR of 2.5 corresponded to a 95% sensitivity and 91% specificity (Figure 2C). The ROC coordinates for whole tumor and tumor margin analyses are listed in eTable 2 in the Supplement.

BLZ-100 Uptake in a Model of Oral Dysplasia

Because BLZ-100 showed high sensitivity and specificity for differentiation of SCC from normal tissue, we next examined its ability to differentiate among the stages of dysplasia. Hamsters were treated with DMBA for 6 weeks (n = 6), 9 weeks (n = 6), or 12 to 16 weeks (n = 15) and subsequently injected with BLZ-100 (Table). An injection-control cohort (n = 6) was treated for the same durations and injected with ICG-free dye. A treatment-control cohort (n = 6) was treated with mineral oil and subsequently injected with BLZ-100.

A total of 78 cheek specimens were analyzed for histologic grading. Cheeks treated with mineral oil (n = 6) and untreated left-sided cheek pouches in all cohorts (n = 39) were histologically normal, without evidence of dysplasia or hyperplasia. In DMBA-treated cheeks (n = 27), we observed all pathologic grades of dysplasia: grade 0 (no dysplasia, n = 4), grade 1 (mild, n = 3), grade 2 (moderate, n = 10), grade 3 (severe, n = 6), and grade 4 (CIS and early SCC, n = 4). The injection-control cohort included grade 1 (n = 2), grade 2 (n = 3), and grade 3 (n = 1) specimens.

While both the mineral oil cohort and grade 0 specimens did not exhibit dysplasia, all grade 0 specimens featured changes in the stratified layers including thickening of the epithelium and stratum corneum. Dysplastic tissue was
characterized by increasing cellular atypia from the basal layer (grade 1) extending up to two-thirds (grade 2) or greater than two-thirds (grade 3) of the epithelium, and marked atypia throughout the entire thickness of the epithelium (grade 4). A few specimens demonstrated gross, exophytic, tumor-like growth with severely atypical histologic features throughout but only the suggestion of early invasion through the basal layer. These specimens were thus classified as early SCC and grouped together with CIS as grade 4 dysplasia. Representative hematoxylin-eosin-stained sections for each dysplasia grade are shown in Figure 3.

We observed selective uptake of BLZ-100 in DMBA-treated cheeks (Figure 4A). SBR was calculated using the contralateral normal cheek as background. In DMBA-treated specimens injected with BLZ-100, SBRs gradually increased with dysplasia severity ($P < .001$) (Figure 4B). There was minimal relative fluorescence uptake in treatment control and injection control cheeks (Table).

To assess whether BLZ-100 uptake was significantly different among high- and low-risk dysplasia categories, we grouped cheek samples as follows: grade 0 ($n = 4$), grade 1/2 (low-risk dysplasia, $n = 13$), and grade 3/4 (high-risk dysplasia, $n = 10$). There was a significant difference in the mean (SD) SBR between histologically normal tissue and grade 0 dysplasia ($1.04 [0.14]$ vs $1.38 [0.26]$; $P = .03$), and between grade 1/2 and grade 3/4 dysplasia ($1.51 [0.34]$ vs $2.31 [0.71]$; $P = .006$) (Figure 4C).

Figure 3. Hematoxylin-eosin–stained Sections Demonstrating the Range of Histopathologic Grades Seen in the Hamster Dysplasia Model

A, Normal tissue, without hyperplasia or hyperkeratosis. Asterisk represents stratum corneum, bracket represents epithelial layer, and curly bracket represents stromal layer.

B, Hyperplasia with hyperkeratosis (asterisk), thickening of the epithelium (bracket), and hyperplasia of the basal layer (arrowheads).

C, Mild dysplasia with hyperkeratosis (asterisk), thickening of the epithelium (bracket), stromal inflammatory infiltrate and multifocal mild dysplasia characterized by increased number of cells in the basal and parabasal epithelial regions with nuclear hyperchromatism and pleomorphism (arrowheads).

D, Moderate dysplasia with hyperchromatism and pleomorphism extending into the lower half of the epithelium (bracket).

E, Severe dysplasia affecting greater than two-thirds of the epithelium.

F, Carcinoma in situ with marked cellular and nuclear pleomorphism and keratin formation (arrowheads).
Discussion

In this study, we evaluated the ability of BLZ-100 to accurately identify the spectrum of HSNCC, from dysplasia to malignant disease.

In our xenograft model, BLZ-100 demonstrated localized uptake that was highly correlative of GFP signal in PCI-15B tumors. BLZ-100 uptake was also evident in xenografts grown from another established cell line (UM-SCC-22B), an HPV-positive cell line (UM-SCC-47), and a low-passage, patient-derived primary culture (FHCRC/UW-SCC1). Compared with established HNSCC cell lines, low-passage, primary cultures have not been subjected to the same clonal selection pressures as established cell lines have and, thus, may more closely reflect the intratumor clonal heterogeneity of clinical tumors. In these tumor xenografts, BLZ-100 uptake was similar to the uptake seen in tumors derived from established cell lines.

In our in situ analysis of fluorescence in tissue sections, BLZ-100 demonstrated highly sensitive and specific uptake at the tumor periphery. To investigate this pattern of uptake, we performed stains of the proliferative marker Ki67 and of the vascular markers CD31/CD34 (eFigure 4 in the Supplement). Ki67 staining was uniform and did not suggest central tissue necrosis. Interestingly, CD31/34 staining was less prominent within the tumor core compared with the surrounding tissue, suggesting that BLZ-100 may have had limited vascular access to the tumor center. Alternatively, the lack of BLZ-100 uptake in the tumor core may reflect an absence of BLZ-100 targets, which may instead be more abundant on the tumor periphery. From the surgical perspective, the enhancement of the tumor periphery may actually be advantageous, because the challenge of complete oncologic resection is not identifying tumor bulk but rather capturing residual disease at the tumor margin.

While we did not primarily intend to visualize metastatic disease, we observed BLZ-100 uptake in a region where metastatic disease was embedded in adventitial tissue (Figure 1E-F). In this case, the SBR of BLZ-100 was higher than the SBR of GFP. This finding may highlight the deeper tissue penetration of near-infrared fluorophores compared with fluorophores with wavelengths similar to that of GFP. Further study is needed to test this hypothesis.
In addition to identifying malignant disease, the detection and treatment of dysplasia are important, because appropriate excision of dysplastic lesions can significantly reduce the risk of malignant transformation.84 However, the currently available tools to identify dysplasia, such as toluidine blue and endogenous tissue fluorescence detection, demonstrate high rates of false-positivity and the inability to distinguish high-risk from low-risk lesions.29-33 Intravenous fluorescent-labeled targeting agents, based on protoporphyrin precursors,34 antibodies,35 and enzyme-activatable peptides,36 represent a promising strategy to deliver accurate, high-resolution identification of tumor with minimal adverse effects. However, there is limited study of these agents within the context of dysplasia27-39, to our knowledge only 1 other agent besides that used in our present study has been reported in a model of oral dysplasia.60 Our results demonstrate that BLZ-100 signal increases with dysplasia severity and notably distinguishes high-risk from low-risk dysplasia. In practice, this distinction may provide clinicians a guide to identify and treat high-risk lesions.

Conclusions

BLZ-100 demonstrates sensitive and specific uptake in HNSCC tumor xenografts. In addition, BLZ-100 uptake increases with dysplasia severity and differentiates between high-risk and low-risk dysplasia. Clinically, BLZ-100 may be useful in sparing unnecessary biopsies or, alternatively, prompting necessary surgery.

REFERENCES


