Published in final edited form as:

J Oral Maxillofac Surg. 2011 September ; 69(9): 2452–2459. doi:10.1016/j.joms.2010.12.041.

Targeted Dendrimer Chemotherapy in an Animal Model for Head and Neck Squamous Cell Carcinoma

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Abstract

Background—Nanoparticle drug delivery offers a potential solution in the treatment of cancer. Using a heterotopic tumor model for head and neck squamous cell carcinoma (HNSCC), tumors of variable folate binding protein- α (FBP- α) have been treated to delineate receptor necessity as well as efficacy and toxicity of folate targeted chemotherapy.

Methods—UM-SCC and ATCC cell lines were screened using quantitative real-time polymerase chain reaction for FBP- α expression. Acetylated generation 5 dendrimers conjugated to the targeting moiety folic acid and the therapeutic moiety methotrexate were fabricated and administered to SCID CB-17 mice inoculated with UM-SCC-1, UM-SCC-17B, and UM-SCC-22B cancer cells. Mice were injected with targeted therapy, free methotrexate, or saline control and monitored for drug efficacy and toxicity.

Results—Targeted therapy was effective relative to receptor level expression. Targeted therapy could be delivered in molar doses 3 times that of free drug. The treatment of a high folate expression tumor cell population was noted to have increased efficacy over saline ($P < .01$) and free methotrexate ($P = .03$) as well as decreased systemic toxicity.

Conclusions—This report represents the first translation of dendrimer-based chemotherapy to HNSCC and underscores its effectiveness as an antitumor agent in human cancer cell lines with lower levels of FBP- α than the in vitro and in vivo models previously reported.

A tremendous need exists for new treatment strategies in the management of squamous cell cancer of the head and neck (HNSCC). It is the sixth most common malignancy in the world and has a mortality rate that is basically unchanged over the past 30 years.¹ In addition, elimination of suffering from this disease will require modalities that not only control cancer but do so in a manner that eliminates systemic toxicity. Current approaches lead to morbidity associated with chemotherapy, the localized destruction of peritumoral tissue via radiation, and the attendant disfigurement associated with surgical therapies. Chemotherapeutic agents that specifically target and concentrate in tumor cells are a feasible and attractive alternative to standard chemotherapy and leave normal tissue unaffected. It is hypothesized that such an approach would reduce systemic toxicity and maintain or increase treatment efficacy.

Advances in nanoparticle drug delivery offer a potential vehicle for targeted therapy. Dendritic noncationic biocompatible polymers (dendrimers) can now be synthesized in large quantities of uniform structure and design and can be conjugated to targeting ligands (Folate, EGF, RGD peptides, etc), imaging agents (Au and Fe), and therapeutics (Taxol, cisplatin, methotrexate, etc)² (Fig 1). Tumors expressing the specific biomarker of the high-affinity folate binding protein- α (FBP- α) have been targeted through this technology, demonstrating a significant increase of therapeutic efficacy and decrease in systemic toxicity.³

We hypothesized that dendrimer targeted therapy would be efficacious and decrease systemic toxicity in an animal model for HNSCC. To test this hypothesis, we 1) screened and identified cell lines with variable expression of the folate receptor target, 2) tested in vivo efficacy of targeted chemotherapy to 3 cell-line xenografts with variable folate receptor expression (no, intermediate, and high expression), and 3) validated the most responsive tumor in a dose-ranging efficacy and toxicity study compared with standard methotrexate.

Materials and Methods

SYNTHESIS AND CHARACTERIZATION OF POLYAMIDOAMINE PAMAM DENDRIMER CONJUGATES WITH FOLATE AND METHOTREXATE FOR TARGETED CHEMOTHERAPY TO HNSCC

A G5 polyamidoamine (PAMAM) dendrimer was synthesized and purified from low molar mass contaminants as well as higher molar mass dimers or oligomers. The synthesis and purification was identical to previously published protocols and performed by the same personnel.³ The average molar mass of the dendrimer was determined to be 26,530 g/mol by size exclusion chromatography using multiangle laser light scattering, ultraviolet, and refractive index detectors. The average number of surface primary amine groups in the dendrimer was determined to be 110 using potentiometric titration along with the molar mass. The polydispersity index, defined as the ratio of weight average molar mass and number average molar mass for an ideal monodisperse sample, equals 1.0. The polydispersity index of G5 dendrimer was calculated to be 1.032, indicating a narrow distribution around the mean value and thus confirming the high purity of the G5 dendrimer. The surface amines of G5 PAMAM dendrimers were acetylated with acetic anhydride to reduce nonspecific binding of the polycationic dendrimer. The ratio between the acetic anhydride and the dendrimer was selected to achieve different acetylation levels from 50 to 80 and 100 primary amines. After purification, the acetylated dendrimer was allowed to react with an activated ester of folic acid, and the purified product of this reaction was analyzed by ¹H nuclear magnetic resonance (NMR) to determine the number of conjugated folic acid molecules. Subsequently, methotrexate was conjugated via ester bond as described previously.⁴ The quality of the PAMAM dendrimer conjugates was tested using polyacrylamide gel electrophoresis, ¹H NMR, ¹³C-NMR, and mass spectroscopy. Capillary electrophoresis was used to confirm the purity and homogeneity of the final products. The folic acid-targeted conjugates specifically contain the following molecules: G5-(Ac)₈₀-(FA) 5-MT $\times$ 5, identified with the acronym G5-FA-methotrexate (MTX).

CELL LINES

UMSCC cell lines were generously supplied by Dr Thomas Carey at the University of Michigan Head and Neck Cancer SPORE. All other lines were obtained directly from the ATCC. Lines were chosen with the goal of obtaining a variable expression pattern of the FBP- α receptor, which we anticipated would be the targeted receptor on the cell for our targeted chemotherapy.

CELL CULTURE

Cell lines were subcultured in 5% CO₂ humidified incubator at 37°C, Corning 75 cm² tissue culture flasks 430641, 15 mL folate-free RPMI 1640 with L-glutamine and phenol red 27016-021 supplemented with Gibco 5% FBS US origin and heat-inactivated 10082-147; 100 U/mL penicillin-streptomycin Gibco 15140-122; 1× final concentration of MEM nonessential amino acids Gibco 11140-050: “Cell Culture Media.” Cell culture media was changed 3 times per week, and the cell lines were passed at ~70% confluence to maintain log-phase growth conditions. Cells lines were subcultured in the previously noted conditions for 10 to 14 days before testing or inoculation into mice. Cell lines were tested for mycoplasma contamination using Myco-Alert assay LT07-218 Cambrex and independently verified by polymerase chain reaction (PCR). Cell lines with mycoplasma contamination were treated with Plasmocin (InvivoGen, San Diego, CA) according to manufacturer’s instructions, followed by retesting before use for study.

QUALITATIVE REAL-TIME PCR

Quantitative real-time (qRT) PCR was used to screen 11 HNSCC tumor cell lines for FBP- α expression with the goal of identifying no expression, intermediate expression, and high expression lines for testing targeted therapy in xenograft animal models. Cepheid SmartCycler (Sunnyvale, CA) conditions: stage 1: 95°C for 120 seconds, stage 2: temp cycle repeat 45 times, 95.0°C for 15 seconds, 66.0°C for 15 seconds, 72.0°C for 25 seconds with optics on, stage 3: melt curve 60.0°C to 95.0°C at 0.2°C/second with optic ch 1 on. Dye: Sybr Green (Invitrogen) reaction conditions: 1.5 ng of RT product, 2.25 μ L 10 × PCR buffer, 2.5 μ L 50 nmol/L MgCl₂, 0.5 μ L 10 mM dNTP, 3’ and 5’ primer 1 μ L each, 1 μ L 1/800 Sybr/TE pH 8.0, 5 μ L 5× additive reagent, 1.25 U platinum Taq (Invitrogen).

Primer: β -actin product size = 500 tgcacctctgtcgcaa sense, tacgcctctggccta antisense; Primer: FBP- α gcagtgtgaatgcaggta sense, acgggtcttctaggcaa antisense. PCR primers are designed to span genomic portions of DNA such that extracted and subsequently transcribed mRNA that is contaminated with genomic RNA results in a 300-bp larger amplicon (800 bp total), whereas a “pure” mRNA extraction results in a 500-bp amplicon. Integrity and specificity of the primer design for folate- α and β -actin are then tested by amplification using PCR. Amplicons are gel extracted and sequenced for their entire lengths of 500 bp. Sequencing results are BLASTed against all known sequences and found to be specific for the proteins of interest. No qRT-PCR data were reported that failed to provide a single, sharp melt curve at the appropriate temperature as provided by the Cepheid software immediately after amplification.

ANIMAL FACILITIES AND ANIMALS

All animal testing was performed under the guidelines and within the facilities of the University of Michigan Unit for Laboratory Animal Medicine in U.S. Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care certified pathogen-free environments. Mice were purchased from Charles River and delivered to the facilities directly. The strain of mice is CB-17 SCID; mice were female with an age range of 29 to 35 days.

XENOGRAFTS

Xenograft tumor growth models were used to demonstrate the efficacy and toxicity of targeted methotrexate chemotherapy versus standard chemotherapy with methotrexate. In our first set of animal experiments, xenografts of no folic acid receptor expression (UM-SCC-22B), intermediate folic acid receptor expression (UM-SCC-1), and high folic acid receptor expression (UM-SCC-17B) were compared with each other with saline control. In

the second experiment, dose ranging of the most sensitive line was tested to validate our hypothesis of increased efficacy and decreased toxicity of targeted therapy compared with standard methotrexate chemotherapy.

Verified mycoplasma free cell lines were inoculated into the hind flanks of 29- to 42-day-old female CB-17 SCID (Charles River) mice in a pathogen-free animal laboratory. The cell lines were sterilely prepared for inoculation with 0.9% injectable sodium chloride USP to a concentration of 4×10^6 per 100- μ L injection site. The hind flank injections were carefully administered so as not to puncture the subdermal tissue, thereby mediating intraperitoneal invasion. The subsequent tumors were measured in millimeter increments by a digital caliper weekly in the x, y, z axes to establish tumor volume and therefore compare the results of treatment by targeted chemotherapy and control standard methotrexate on tumor growth; treatment was initiated when the tumors reached a size of approximately 100 mm³ per inoculation site, this size was achieved in 7 to 10 days.

DELIVERY OF TARGETED NANOPARTICLE THERAPEUTIC

Five times per week (daily Monday through Friday) SCID mice with UM-SCC xenografts received injections of either dendrimer targeted MTX, free MTX, or saline control based on previously identified daily dosages with known toxicity profiles in the animal model. The compounds were delivered in a 0.2-mL volume of saline. Molar ratios of MTX were calculated for all doses delivered according to the number of MTX molecules present in each nanoparticle. In each trial, up to 6 mice per group based on statistical design with significant *P* value set at 0.05 received a total of 20 injections (dependent on survival). The body weights and tumor volume of the mice were monitored twice per week as an indication of positive and adverse effects of the drug. Histopathology of the tumor and multiple organs was done at the termination of the trial with tissues from the tumor, lung, heart, liver, spleen, kidney, intestine, brain, and muscle examined.

STATISTICAL METHODS

Data were placed into SPSS software for statistical analysis. Means, SD, and SEM of the data were calculated. Differences between the experimental groups and the control groups were tested using Student's-Newman-Keuls' test, and *P* values less than .05 were considered statistically significant.

Results

QRT-PCR

Using our primers for FBP- α , 11 HNSCC cell lines (Table 1) were screened for comparison of FBP- α expression. Of these lines, HNSCC lines showed low levels of expression compared with other tumor types known to express FBP- α . Specifically related to KB cells that had been studied previously, the expression of FBP- α in HNSCC was 1000-fold less. HNSCC lines did reveal variable expression patterns of FBP- α , with UM-SCC-22B having no expression and UM-SCC-17B with the highest level of expression (Fig 2).

ANIMAL MODEL STUDIES

On the basis of qRT-PCR results, 3 cell lines were chosen for study using a relatively no, intermediate, and high FBP- α expressing cells considering only HNSCC lines. This study confirmed our hypothesis that folate expression was relevant to targeted therapy tumor responsiveness. Compared with saline control, targeted therapy had no effect on UM-SCC-22B, intermediate effect on UM-SCC-1, and strong effect on UM-SCC-17B (Fig 3).

With the necessity of receptor expression for targeted therapy confirmed, we explored further the effects of targeted therapy in the highest FBP- α expressing HNSCC line UM-SCC-17B using high and low doses of free MTX and targeted therapy versus saline control. In this study, we demonstrated the ability to deliver targeted MTX at doses in molar doses 3 times the dose of free MTX (considering equimolar molar ratios) with significant increases in tumor control efficacy (Fig 4) and decreases in systemic toxicity (Fig 5). For example, 3.33 mg/kg/d free MTX was lethal at 2 weeks, whereas the animals receiving 160 mg/kg/d targeted therapy survived the 28-day trial. Both low- and high-dose targeted therapy demonstrated tumor control better than saline or free MTX, but only high-dose targeted therapy demonstrated a statistically significant increase in tumor control versus saline ($P < .01$) and free MTX ($P = .03$).

Discussion

In this study, we tested the hypothesis that FBP- α was found in HNSCC and could therefore be targeted similar to previously published results in vitro and in vivo in KB cells. We validated our hypothesis by identifying cell lines with variable expression of FBP- α , demonstrating varying degrees of tumor control attributable to receptor expression in a xenograft mouse model and validating increased efficacy and decreased toxicity compared with standard MTX chemotherapy. This report represents the first translation of dendrimer targeted MTX chemotherapy to an animal model for HNSCC and has many important ramifications for this emerging technology.

From a folic acid receptor perspective, significant differences exist between KB cells and UM-SCC-17B, which were validated in this project. KB cells are known to highly overexpress FBP- α , especially in the face of folate deprivation. In contrast, UM-SCC-17B, although the highest of any of the UM-SCC cell lines we tested, expresses FBP- α at mRNA levels approximately 1000-fold less than KB and does not upregulate FBP- α in response to low folate levels even when these levels are maintained over weeks to months (data not shown). Pertinent to this, in our study model, we did not require folate deprivation of animals to demonstrate drug effect.

We used mRNA levels to predict efficacy of targeted therapy given that escalating antitumor effects correlated with mRNA expression of FBP- α . Whether mRNA levels correlate exactly to receptor number and activity in the chosen cell lines is unknown given post-translational modifications and receptor kinetics. Previous testing in our laboratory has demonstrated similarities in mRNA level and receptor activity for HNSCC lines using radiolabeled assays but was not specifically addressed for each of the lines in this study. Regardless, our results here support the concept that mRNA testing may have predictive value in identifying potential responders to this therapy. This would be particularly helpful given that not all HNSCC tumors overexpress FBP- α and intermediate expression did not offer a significant tumor control advantage. If further validated, one could envision a clinical scenario in which biopsies could be screened for upregulated receptor levels, guiding the targeting agent of choice with the possibility of multiple targets, with dendrimers providing an interchangeable platform. At minimum, our work supports the concept that qRT-PCR could be used to select patients who would not be amenable to an FBP- α targeted approach because of their absolute lack of FBP- α for targeting.

Two important additional findings in our study should be noted. First, 1 mouse in the low-dose treatment group had near complete tumor regression in the 30-day trial. This result is similar to the previously published results with KB in which the only mice that survived a 100-day trial were those treated with targeted therapy.³ In that study, 2 mice were deemed complete responders, whereas others in the same group continued to experience tumor

growth. Second, in our studies, equimolar tumor effective doses were less toxic than free drug, similar to the results of previous studies. One of the significant advantages reported with targeted therapy is decreased systemic toxicity. Given tumor control, if systemic toxicity can be limited, then morbidity and human suffering associated with disease treatment can be decreased.

Dendrimer targeted approaches offer great promise to cancer therapy overall. Our results suggest that dendrimer targeted therapy is feasible in an animal model for cells that overexpress FBP- α at the mRNA message level even if these levels are far below the previous model in KB cells. This result is significant because it has the potential to dramatically increase the number of tumor types that could be treated with folate targeted chemotherapy. KB tumor characteristics of extremely high levels of FBP- α overexpression are not required for clinical effect and are not necessary to achieve decreased systemic toxicity. Our findings with the UM-SCC-17B cell line demonstrate that differential targeting can be had at more modest levels of FBP- α overexpression.

A large number of biomarkers have been identified and are under study, underscoring the numerous possibilities for targeted therapy with a dendrimer-based approach. Looking at HNSCC alone, Chin and Boyle et al analyzed the expression of more than 13,000 genes that gave rise to 1260 with differential expression in HNSCC, including many cell surface receptors (EGF receptor, interleukin-1 receptor, insulinlike growth factor binding protein, thyroid hormone receptor, and integrin alpha-6).⁵ Although these markers may not in themselves be transforming, it is hypothesized that they are overexpressed in response to the metabolic demands of a persistently dividing cell. Given the presence of differentially expressed biomarkers in rapidly dividing cells, it is suggested that targeting of these receptors may also be possible as a substitute or in conjunction with FBP- α .⁶⁻⁸

Methotrexate was chosen as the therapeutic in this study and has been demonstrated to be an effective for treatment of HNSCC in preclinical and clinical settings. Like many chemotherapeutic agents for cancer, systemic toxicity significantly limits its application. Although other therapies have now superseded MTX as primary treatment, the possibility of delivering targeted MTX while decreasing overall systemic toxicity could potentially overcome the previous limiting nature of the therapeutic and was part of the rationale for the therapy choice. In addition, alternative dendrimer conjugates have been created with Taxol,⁹ docetaxel, and cisplatin with plans for a number of additional therapeutics being considered for future development.

Recent advances in directed chemotherapy for cancer have demonstrated the ability to deliver chemotherapy in vitro and in vivo with increased efficacy and decreased toxicity over standard chemotherapeutic approaches in a number of cancers, including lung cancer, 10 colon cancer,¹¹ and head and neck cancer.¹² It is likely that with continued development, targeted approaches will become the mainstay of cancer therapy. Dendrimer-based therapy has great potential in this regard because of the ability to conjugate alternative targeting agents and therapeutics to a single carrier device. A number of alternative targets are under exploration using dendrimer-based approaches, which may allow us to modify therapeutic modalities (both the receptor targeted and therapeutic utilized) in the future according to the genetic makeup of the tumor characteristics. This study highlights a mechanism for clinical application by the selection of a tumor for therapy based on mRNA expression of the desired target. In the future, screening of tumors for a large number of potential targets could be possible with dendrimer targeted therapy, selected specifically on the basis of receptor expression and tumor responsiveness to drug. We are currently continuing our animal work to delineate the therapeutic index of this therapy more effectively and to expand our research to other receptors for targeting.

Acknowledgments

This work was supported by the University of Michigan Head and Neck Cancer SPORE (NIH/NCI Grant No. CA97248-01).

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Computer Model of a Multi-functional PAMAM Dendrimer

FIGURE 1.
Computer model of the nanodevice.

FIGURE 2.

Quantitative real-time polymerase chain reaction of 11 cell lines for folate binding protein- α sorted on the basis of relative fold expression. KB expression (not shown) extends beyond the graphed portions to a relative fold of 29.86.

FIGURE 3.
Tumor control model in xenograft mice with UM-SCC-1, 17B, and 22B, delivered targeted methotrexate versus saline control.

FIGURE 4.
Tumor control in xenograft mice with UM-SCC-17B delivered targeted methotrexate (MTX) versus saline and free MTX.

FIGURE 5.
Mean animal weights demonstrating systemic toxicity in UM-SCC-17B xenograft mice delivered targeted methotrexate (MTX) versus saline and free MTX.

Table 1
Cell line characteristics for studies with quantitative real-time polymerase chain reaction

Line Name	Type	Source	Gender	Age	Organ
Um-SCC-1	HNSCC	UMSCC	Male	73	Floor of mouth
Um-SCC-6	HNSCC	UMSCC	Male	37	Base of tongue
Um-SCC-11B	HNSCC	UMSCC	Male	65	Hypopharynx
Um-SCC-14A	HNSCC	UMSCC	Female	58	Floor of mouth
Um-SCC-17B	HNSCC	UMSCC	Female	47	Neck
Um-SCC-22B	HNSCC	UMSCC	Female	58	Neck
Um-SCC-74A	HNSCC	UMSCC	Male	50	Base tongue
Um-SCC-81B	HNSCC	UMSCC	Male	53	R. true Cord
SCC-15	HNSCC	ATCC	Male	55	Tongue
SCC-25	HNSCC	ATCC	Male	70	Tongue
FaDu	HNSCC	ATCC	Male	56	Pharynx

Abbreviation: HNSCC, head and neck squamous cell carcinoma.