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Novel C-Terminal Heat Shock Protein 90 Inhibitors (KU711 and Ku757) Are Effective in Targeting Head and Neck Squamous Cell Carcinoma Cancer Stem cells

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Abstract

Advanced head and neck squamous cell carcinoma (HNSCC) remains a therapeutic challenge due to the development of therapy resistance. Several studies have implicated the development of cancer stem cells as a possible mechanism for therapy resistance in HNSCC. Heat shock protein 90's (Hsp90's) molecular chaperone function is implicated in pathways of resistance in HNSCC. Therefore, in the present study, we investigated the efficacy of novel C-terminal Hsp90 inhibitors (KU711 and KU757) in targeting HNSCC cancer stem cells (CSCs). Treatment of HNSCC human cell lines MDA1986, UMSCC 22B, and UMSCC 22B cisplatin-resistant cells with the KU compounds indicated complete blockage of self-renewal for the resistant and parent cell lines starting from 20 μM KU711 and 1 μM KU757. Dose-dependent decrease in the cancer stem cell markers CD44, ALDH, and CD44/ALDH double-positive cells was observed for all cell lines after treatment with KU711 and KU757. When cells were treated with either drug, migration and invasion were downregulated greater than 90% even at the lowest concentrations of 20 μM KU711 and 1 μM KU757. Western blot showed >90% reduction in client protein "stemness" marker BMI-1 and mesenchymal marker vimentin, as well as increase in epithelial marker E-cadherin for both cell lines, indicating epithelial to mesenchymal transition quiescence. Several CSC-mediated miRNAs that play a critical role in HNSCC therapy resistance were also downregulated with KU treatment. *In vivo*, KU compounds were effective in decreasing tumor growth with no observed toxicity. Taken together, these results indicate that KU compounds are effective therapeutics for targeting HNSCC CSCs.

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Introduction

Despite the introduction of newer therapeutic protocols, mortality rates associated with head and neck squamous cell carcinoma (HNSCC) have remained largely unchanged over the last four decades [1]. Concurrent radiation and chemotherapy have become the standard for adjuvant therapy after surgical ablation, as well as the definitive treatment of HNSCC in select cases. Systemic platinum-based chemotherapy, namely, cisplatin, remains a first-line agent due to its radiosensitizing and cytotoxic effects [2,3]. Unfortunately, chemotherapy and radiation-based treatments have been associated with significant toxicity, particularly in patients receiving concurrent chemoradiotherapy [4]. HNSCC has shown

Abbreviations: ALDH, aldehyde dehydrogenase; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; CSC, cancer stem cell; EMT, epithelial to mesenchymal transition; HNSCC, head and neck squamous cell carcinoma; HSP, heat shock protein (Hsp90, Hsp70); KU, Kansas University (compounds KU757, KU711); SCC, squamous cell carcinoma; UMSCC, University of Michigan Squamous Cell Carcinoma (cell lines UMSCC 22, UMSCC 22B-cis); WB, Western blot.

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marked resistance to radiation and cisplatin in many cases, and treatment resistance requiring dose escalation and resultant toxicities continues to be problematic, highlighting a need for the development of novel therapies that effectively treat this disease and its cisplatin resistance [5,6].

Cancer stem cells (CSCs) represent a subpopulation of cells within a tumor that have the ability of self-renewal and regeneration. Recent literature suggests that this population of cells is thought to significantly contribute to tumor proliferation, invasion, metastatic potential, and resistance to drug therapy [7–11]. This subpopulation may lie quiescent for periods of time as well as harbor protective mechanisms against cellular damage, and is felt to be responsible for the majority of tumor growth and metastatic potential in HNSCC [11,12]. Furthermore, CSCs have been shown to contribute to resistance against chemotherapeutic agents, including platinum-based regimens along with external beam radiation [5,13]. Nor et al. using an HNSCC xenograft model observed that cisplatin treatment increased the fraction of CSCs as defined by the $ALDH^{high}/CD44^{high}$ populations, again implicating this cellular subpopulation in resistance to current standard-of-care therapies [14]. Thus, it seems to reason that in order to more effectively treat or abrogate chemo- and drug resistance in HNSCC, this process should involve some level of targeting of the CSC population.

Heat shock protein (Hsp) 90 is a molecular chaperone protein that regulates several "client" proteins involved in cancer development, including proteins involved in pathways critical for cell growth, invasiveness, and survival [15]. Numerous proteins implicated as critical for CSCs' development are also dependent on Hsp90. This suggests a high therapeutic potential for Hsp90 inhibitors as they can simultaneously suppress multiple oncogenic pathways involving the bulk tumor cell population of a cancer as well as its CSCs. Use of first- and second-generation Hsp90 inhibitors targeting the N-terminal domain of the chaperone was restricted due to dose-limiting toxicity, resulting mainly from activation of the heat shock response leading to induction of compensatory proteins (e.g., Hsp70) with prosurvival effects [16]. Thus, early-generation N-terminal Hsp90 inhibitors have not progressed beyond early-phase clinical trials despite showing potent anticancer effects. To address limitations of N-terminal Hsp90 inhibitors, our group has developed potent, novel Hsp90 inhibitors targeting the carboxy terminus of the chaperone which blocks Hsp90 chaperone function without concurrently upregulating Hsp70 and its prosurvival effects, thus avoiding this key limitation of N-terminal inhibitors [17–21]. These compounds have potential to act synergistically with current standard-of-care therapies and prolong or prevent development of drug resistance [16,22]. Hence, we hypothesized that C-terminal Hsp90 inhibitors (especially our lead compounds KU711 and KU757, chosen for their potency and selectivity for cancer cells; structures in Supplemental Figure 1) can inhibit key CSC functions including migration, invasion, self-renewal, and epithelial to mesenchymal transition (EMT); can target the miRNAs involved in CSC function; and can reduce tumor growth of HNSCC xenografts.

Materials and Methods

Cell Culture

The validated HNSCC cell line UMSCC 22B was generated at the Michigan Otolaryngology and Translational Oncology Laboratory and graciously donated by Dr. Thomas Carey. UMSCC 22B-cis, a kind gift from Dr. Jacques Nor, University of Michigan, Ann Arbor, is a cisplatin-resistant line generated by co-culturing UMSCC 22B with increasing concentrations of cisplatin *in vitro* up to 12 μM concentration, as previously described [14]. The validated HNSCC cell line MDA-1986 was graciously donated by Dr. Jeffrey Myers (University of Texas, M.D. Anderson Cancer Center, Houston, TX). Cells were grown and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% combination of penicillin and streptomycin (Sigma-Aldrich) in a 37°C humidified atmosphere of 5% CO_2 in air. Drug compounds used in these experiments included two C-terminal Hsp90 inhibitors, KU711 and KU757, and these were obtained from Dr. Brian S. J. Blagg (University of Notre dame, Indiana, IN). Finally, a standard N-terminal Hsp90 inhibitor, 17-AAG, was obtained from Sigma-Aldrich.

Orosphere Formation Assay

MDA-1986, UMSCC 22B, and UMSCC 22B-cis cells were plated at 100 cells/well in a 96-well ultralow attachment plate (Corning, Corning, NY) in low-glucose DMEM (Life Technologies) with varying concentrations of KU711, KU757, 17-AAG, and cisplatin [up to five times their half-maximal inhibitory concentration (IC50) values[. Cells were cultured for 10 to 14 days, and orosphere formation (>25 cells) was assessed in each well using light microscopy.

ALDEFLOUR Assay/Flow Cytometry

UMSCC 22B, UMSCC 22B-cis, and MDA-1986 cells were treated and collected as described for Western blot analysis and evaluated for ALDH activity using the ALDEFLOUR assay kit as per the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). CD44-positive populations were detected by flow cytometry using APC conjugated CD44. Flow cytometric analysis was conducted on a CyAn ADP analyzer (Beckman Coulter, Brea, CA). DAPI staining was used to exclude dead cells from analysis.

Western Blot/EMT

Cells were grown to 60% to 80% confluence and treated for 24 hours at 20 to 40 μM KU711 or 1 to 2.5 μM KU757 (1-2× and 1-2.5× IC50), 1 μM 17-AAG, or 2 μM cisplatin. Treated cells were collected and resuspended in lysis buffer, and protein concentrations were determined using the BSA protein assay (Thermo Fisher Scientific, Waltham, MA). Immunoblotting was performed by methodology published previously [23]. BMI-1, E-cadherin, and vimentin antibodies were purchased from Cell signaling Technology (CST, Danvers, MA), β-actin from Millipore (EMD Millipore, Billerica, MA), and donkey anti-rabbit IgG HRP (1: 3000) and goat anti-mouse IgG HRP (1: 3000) secondary antibodies from Santa-Cruz. Membranes were developed with either SuperSignal West PICO or FEMTO (Thermo Fisher Scientific, Waltham, MA) for 5 minutes and visualized by enhanced chemiluminescence and captured on autoradiography film (Molecular Technologies, St. Lewis, MO) on a Konica Minolta SRX 101A developer (Ramsey, NJ). Actin levels were assessed as a housekeeping gene to ensure equal loading and transfer of proteins. Studies were replicated for accuracy.

Migration and Invasion Assay

UMSCC 22B and UMSCC 22B-cis cell lines were collected and resuspended in serum-free DMEM with penicillin/streptomycin and 20 to 40 μM KU711 or 1 to 2.5 μM KU757 or 1 μM 17-AAG or 2 μM cisplatin. Equal numbers of cells were plated onto either standard