

Mitochondrial Stress–Mediated Targeting of Quiescent Cancer Stem Cells in Oral Squamous Cell Carcinoma

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Introduction: Despite improved therapeutics in oral squamous cell carcinoma (OSCC), tumor cells that are either quiescent and/or endowed with stem cell–like attributes usually survive treatment and recreate tumor load at relapse. Through this study, we aimed strategically to eliminate these stem cell–like cancer cells using a combination drug approach.

Methods: Primary cultures from 15 well–moderately differentiated OSCC were established, and the existence of cancer cells with stem cell–like characteristics using five cancer stem cell (CSC) specific markers — CD44, CD133, CD147, C166, SOX2 and spheroid assay was ascertained. Next, we assessed quiescence in CSCs under normal and growth factor–deprived conditions using Ki67. Among several gene signatures regulating quiescent cellular state, we evaluated the effect of inhibiting Dyrk1b in combination with topoisomerase II and histone deacetylase inhibitors in targeting quiescent CSCs. Multiple drug-effect analysis was carried out with CompuSyn software to determine combination-index values.

Results: We observed that CD44⁺CD133⁺ showed the highest level of SOX2 expression. CSCs showed varying degrees of quiescence, and inhibition of Dyrk1b decreased quiescence and sensitized CSCs to apoptosis. In the drug-combination study, Dyrk1b inhibitor was combined with topoisomerase II and histone deacetylase inhibitors to target quiescent CSCs. In combination, a synergistic effect was seen even at a 16-fold lower dose than IC₅₀. Furthermore, combined treatment decreased glutathione levels and increased ROS and mitochondrial stress, leading to increased DNA damage and cytochrome c in CSCs.

Conclusion: We report marker-based identification of CSC subpopulations and synergy of Dyrk1b inhibitor with topoisomerase II and HDAC inhibitors in primary OSCC. The results provide a new therapeutic strategy to minimize quiescence and target oral CSCs simultaneously.

Keywords: oral cancer, cancer stem cells, drug combination, synergy, apoptosis

Introduction

Oral squamous cell carcinoma (OSCC) is an invasive head–neck malignancy with a 5-year survival rate of <50%. It is frequently associated with recurrences and locoregional and distant metastases. Although advances in therapeutic strategies have helped in achieving high rates of remission, sustaining disease-free status has been difficult to obtain. This is mainly due to intratumor heterogeneity, to which the major contributing factor is cancer stem cells (CSCs).¹ Over the past decade, studies focusing on CSCs in tumors have been rolling in regularly to illustrate their role in tumor development and progression and the clinical implications of targeting these cells. It is now conceded that

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the existence of CSCs portends tumorigenic potential and therapeutic resistance and increases the likelihood of relapse. The ability to eliminate CSCs efficiently depends upon identification of their distinctive surface markers and optimal therapeutic strategies.^{2–4} However, CSCs cannot be defined based on the expression of a single specific marker,⁵ which makes cancer treatment even more challenging.

An additional challenge is slowly dividing or nondividing quiescent tumor cells.⁶ Increasing evidence suggests that cancer cells endowed with stem cell-like characteristics adopt a quiescent phenotype as a survival strategy. Several gene signatures, such as *NR2F1*, *P21CDKN1A*, *PLK1*, and *DYRK1B*, have been identified as regulating the quiescent cellular state.⁷ Either their expression or inactivation is critical in governing transition between cell proliferation and quiescence. A member of the Dyrk family of protein kinases, Dyrk1b is a drugable target regulating G₀/G₁-S phase transition. Dyrk1b confers a survival advantage to transformed and untransformed cells by modifying cell-cycle regulators and helping to maintain them in a quiescent (G₀) state.⁸ It is expressed at low levels in most tissue types and is transcriptionally upregulated in quiescent cells.⁹ It modulates the cell cycle by preventing degradation of p27, while it destabilizes cyclin D and promotes its proteolysis.^{10,11} Therefore, inhibition of Dyrk1b would force quiescent tumor cells into the cell cycle, providing opportunity to target them efficiently.

In this study, we evaluated the effect of the topoisomerase II inhibitor (Topo-i) mitoxantrone (MX) and histone deacetylase inhibitor (HDAC-i) mocetinostat (MO) with the Dyrk1b inhibitor (Dyrk1b-i) AZ191 (AZ). Topo-i is known to inflict damage to rapidly proliferating cells by intercalating in DNA. In combination treatment, Dyrk1b inhibition would bring cells into the cycle, while Topo-i would target these proliferating cells. Furthermore, we also evaluated the combined effect of inhibiting Dyrk1b and HDAC, as HDAC modulates expression of several genes, particularly cell-cycle regulators and tumor suppressors. Given the antitumor effects of inhibiting HDAC alone in solid tumors provides limited therapeutic benefits,^{12,13} its use as part of combination treatment could be more effective. We established primary cultures from histopathologically diagnosed cases of OSCC and evaluated the expression of CSC-specific surface markers² — CD44, CD133, CD147, and CD166 — and the pluripotent stem-cell marker SOX2. Thereafter, we investigated the effect of Dyrk1b-i with Topo-i and HDAC-i in targeting oral CSCs. This combination approach showed synergistic effects and promising results in OSCC.

Methods

Primary Cell Culture

This study was approved by the Institutional Ethics Committee (1057) of King George's Medical University, Lucknow, India. Written informed consent was obtained from all participants included in the study prior to collection of tumor tissue. Single-cell suspensions from tumor samples were prepared as described previously.^{14,15} Briefly, tumor samples were collected in sterile Dulbecco's PBS (Sigma-Aldrich, USA). Connective tissue was carefully removed and tumorous parts minced to obtain 1–2mm³ tissue, followed by enzymatic dissociation. The digested tissue was filtered through a cell strainer. Single-cell suspensions were suspended in MEM supplemented with 10% FBS (Fisher Scientific), 0.5% amphotericin B, and 1% penicillin–streptomycin and cultured in a humidified incubator at 37°C with 5% CO₂. Media were changed every 3–4 days and cells passaged at 85%–90% confluence.

Assessing Expression of CSC Markers

After establishment of cultures, the epithelial nature of primary OSCC cells was determined between passages 1 and 3 by staining with the epithelial cell marker pancyokeratin (pan-CK) (Exbio, Czech Republic). Briefly, after trypsinization 2×10⁵ cells were fixed using a fixation/permeabilization solution kit (Cytofix/Cytoperm; BD Biosciences, USA) for 20 mins at 4°C. Cells were washed twice in BD Perm/Wash buffer and stained with pan-CK antibody overnight at 4°C. The next day, cells were washed twice with BD Perm/Wash buffer before flow-cytometry analysis.

Subpopulations of CSCs were labeled using fluorochrome-conjugated antibodies — CD44 PE-Cy7, CD133 BV421, CD147 BV510, CD166 Alexa Fluor 647 (BD Biosciences) and the pluripotent stem-cell marker SOX2 PE (BioLegend, USA). Briefly, 2×10⁵ primary OSCC cells were stained with aforementioned CD-marker antibodies for 60 minutes in the dark on ice. After staining for surface markers, cells were washed with staining buffer (PBS with 1% BSA) and stained with anti-SOX2.

Ki67 Expression under Normal and Serum-Deprived Conditions

Under normal conditions (serum 10%), cells were seeded at a density of 3×10⁴ cells/well in 12-well flat-bottom tissue culture-treated plates. After 24 hours, staining for CSC surface-marker antibodies and purified mouse anti-Ki67 tagged with Alexa Fluor 488 (BD Biosciences) was carried out to assess

the difference in Ki67 expression in the whole cancer-cell population and CSC subpopulation. Thereafter, we sought to evaluate the effect of serum deprivation (2%) and Dyrk1b inhibition on Ki67 expression. At 24 hours after cell seeding, cells were deprived of serum, and AZ at IC₂₀ concentration was added for 36–48 hours before Ki67 staining.

Cell-Viability Assay and Drug-Combination Analysis

Sulforhodamine B assay¹⁶ was used to assess the cytotoxicity of drugs. Optical density was measured with a SpectraMax M5e (Molecular Devices, USA). Percentage cell growth was calculated as (absorbance sample/absorbance control) × 100. Percentage cell-growth inhibition was calculated as 100 – % cell growth. Tumor cells were initially exposed to individual drugs — MX, MO, and AZ (Cayman Chemical, USA) — to determine half-maximal inhibitory concentration (IC₅₀), followed by combination treatment. Experiments were carried out in non-constant ratio combinations, where AZ was kept at constant IC₂₀ and IC₄₀ concentrations, while concentrations of MX and MO varied from four fold higher to 16-fold lower concentrations of IC₅₀. The effect of drug combinations was evaluated using CompuSyn version 1.0 (ComboSyn, Paramus, NJ, USA) and combination index (CI) values calculated for each concentration of drug interaction to determine synergy (CI < 1.0), additive effect (CI = 1.0–1.1) or antagonism (CI > 1.1).^{17,18}

Spheroid Assay

Tumor spheroid-formation assay¹⁹ was carried out to evaluate the effect of drug combinations. Initially, single-cell suspensions of primary OSCC culture were prepared as described earlier. After determination of cell density, an equal number of cells (2,000 cells/well) in 500 µL medium was seeded in 12-well ultralow-attachment plates (Corning Life Sciences, USA). After 24 hours, IC₂₅ values of MX and MO with AZ IC₁₀ were added. Number, size, and quality of spheroids were assessed under inverted microscopy after 5–7 days.

γH2AX and Apoptosis Assays

Apoptosis was determined using an annexin V-FITC apoptosis-detection kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Apoptotic index values for CSCs were calculated as percentage of apoptotic cells divided by total number of cells.²⁰ Cells were seeded in 12-well flat-bottom tissue culture-treated plates at a density of 3 × 10⁴ cells/well. After 24 hours, MX and MO IC₅₀

were added alone and in combination with AZ IC₂₀. After treatment, cells were trypsinized and stained with surface-marker antibodies. Thereafter, cells were suspended in binding buffer and incubated with annexin V-FITC conjugate for 20 minutes in the dark at room temperature before conducting flow cytometry. Nuclear sites of DNA damage were evaluated with γH2AX expression²¹ for DNA double-strand breaks using Alexa Fluor 488-conjugated purified mouse anti-H2AX (BD Biosciences). After staining with surface-marker antibodies, staining for γH2AX was performed.

Measurement of Mitochondrial Activity, Reactive Oxygen Species, and Cytochrome c Levels

Primary OSCC cells were exposed to drugs for 24 hours and stained with (a) MitoTracker deep red 25 nm for 20 minutes, (b) JC1 (mitochondrial membrane-potential probe) 2 µM for 30 minutes, (c) ThiolTracker violet (glutathione-detection reagent) 2 µM for 20 minutes, and (d) CellRox deep red (cellular oxidative stress detection) 2.5 µM for 30 minutes, as per the manufacturer's instructions (Thermo Fisher Scientific). Cells were washed with PBS and analyzed immediately. Levels of cytochrome c were assessed at 24 hours after combination treatment using anti-cytochrome c antibody (BioLegend).

Flow-Cytometry Acquisition and Analysis

Data acquisition for CSC markers, Ki67 expression, apoptosis analysis, γH2AX expression, and measurement of mitochondrial membrane potential, glutathione, ROS, and mitochondrial mass were done with an FACS Aria III cytometer (BD) and analyzed with FlowJo version 10 (Tree Star, USA).

Statistical Evaluation

Statistical significance for differences among groups was determined by unpaired *t*-tests. *p* ≤ 0.05 was considered statistically significant.

Results

Immunophenotypic Characterization of Oral CSCs

The adherent culture of primary OSCC cells exhibited a characteristic epithelial morphology. Their epithelial nature was demonstrated by positive staining with pan-CK (Figure 1A). Thereafter, we did marker-based characterization of stem cell-like cancer populations. All cases

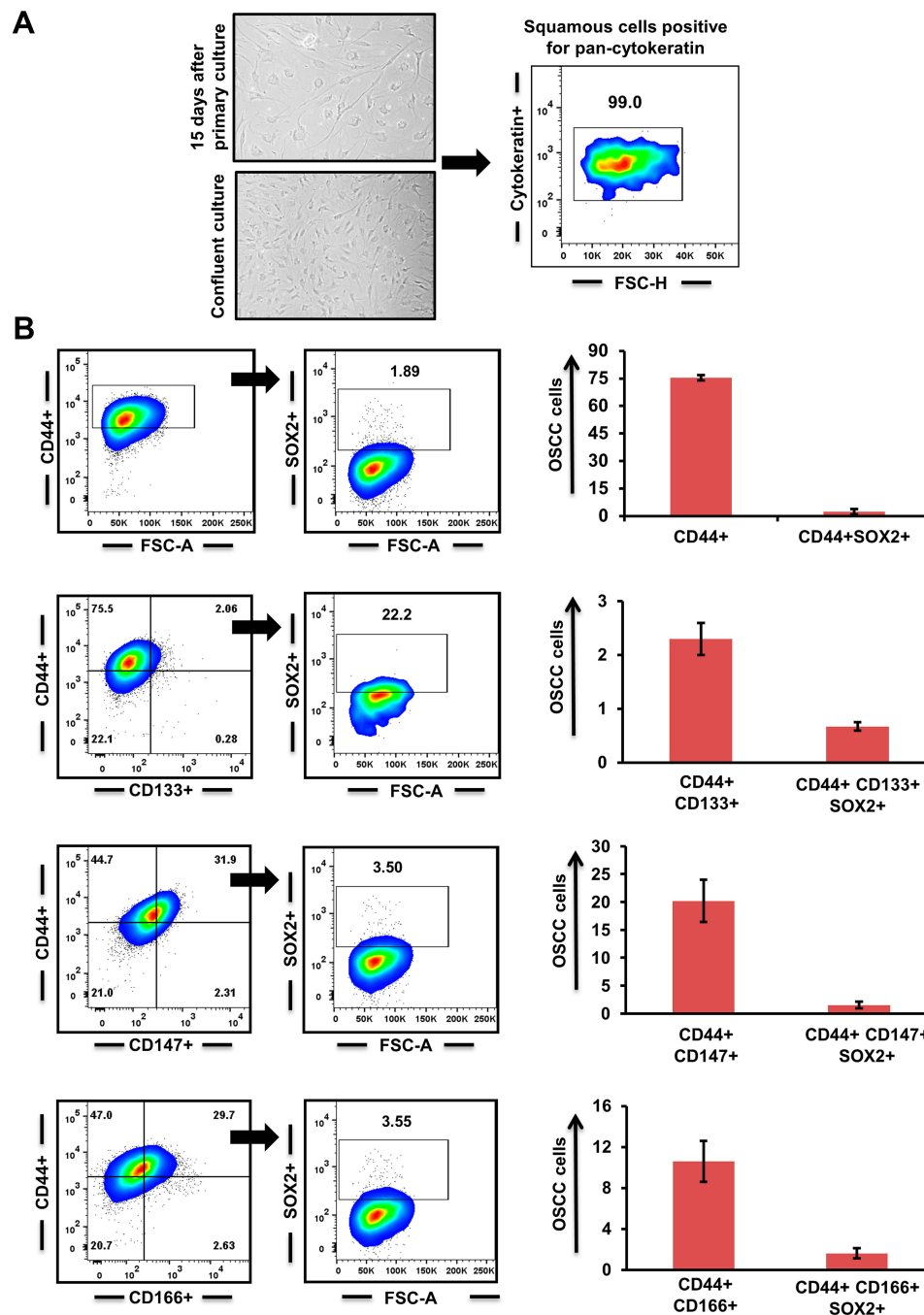


Figure 1 Cancer stem cells (CSCs) in primary oral squamous-cell carcinoma (OSCC) (n=15). **(A)** Primary culture of OSCC cells and characterization of their epithelial lineage with pancytokeratin. **(B)** Surface markers CD44, CD133, CD147, and CD166 used to identify CSC population and Sox2 expression in double-positive (CD44⁺CD133⁺; CD44⁺CD147⁺; CD44⁺CD166⁺) tumor cells.

showed moderate–high expression for CD44, while expression for other markers (CD133, CD147, and CD166) varied from mild to moderate. As CD44 expression is known to be an important regulator of cancer stemness,^{22–24} we analyzed the expression of Sox2 in CD44⁺ cells. Only a small percentage of cells coexpressed CD44 and Sox2. Further, we gated CD133⁺, CD147⁺, and

CD166⁺ cells from CD44⁺ cells and assessed expression of Sox2 in double-positive (CD44⁺CD133⁺, CD44⁺CD166⁺, CD44⁺CD147⁺) cells. Interestingly, we found that the percentage of cells expressing Sox2 was higher in double-positive cells than CD44⁺ cells alone. CD44⁺CD133⁺ cells showed the highest expression for Sox2, followed by CD44⁺CD166⁺ and CD44⁺CD147⁺ (Figure 1B).

Exit from Quiescence Post-AZ Treatment

Coexpression analysis of CSC subpopulations was carried out with Ki67 for analyzing the fraction of cells present in the active phase of the cell cycle. We observed that within the entire population, Ki67 was predominantly expressed in CD44⁺CD133⁺ cells compared to CD44⁺CD147⁺ and CD44⁺CD166⁺ subpopulations (Figure 2A). Further, we encouraged quiescence by serum starvation (2% for 36–48 hours) and added AZ to half the serum-deprived wells. Percentages of quiescent cells in serum-starved culture with and without AZ were demonstrated by negative labeling of Ki67. We observed that a significant percentage of CSCs ($p \leq 0.05$) exited quiescence when Dyrk1b was inhibited (Figure 2B). On serum deprivation, approximately 60% of CD44⁺CD133⁺ cells exhibited a decrease in Ki67 expression, while 74% of CD44⁺CD147⁺ and 43% of CD44⁺CD166⁺ cells showed a quiescent phenotype. On the other hand, when under similar conditions Dyrk1b was inhibited, only around 32% of CD44⁺CD133⁺, 27% of CD44⁺CD147⁺, and 35% of CD44⁺CD166⁺ cells showed negative labeling for Ki67. These data suggest that inhibiting Dyrk1b activity in OSCC may help to overcome the main barrier of quiescence in effective elimination of CSCs.

Dyrk1b Inhibitor Synergized with Topoisomerase II and HDAC Inhibitors

We investigated the effect of each drug alone and in combination. Primary OSCC cells displayed considerable response to MX and MO. Approximate IC₅₀ of MX and MO was 0.33 μM and 4.5 μM, respectively, while that of AZ was 10.9 μM (Supplementary Figure 1A). After determining effects of individual drugs, interaction between MX and MO with AZ IC₂₀ and IC₄₀ was evaluated (<https://www.dovepress.com/get-supplementary-file.php?f=252292.docx>; Figure 3, A and B).

The sensitivity of primary OSCC cells to MX and MO increased considerably when combined with AZ. Potent anti-proliferation effects were seen even at doses <IC₅₀ for MX and MO. Analysis of the combined effect was performed using CompuSyn software based on the median-effect principle. The representative Fa-CI plot for the combination effect is illustrated in Figure 3, C and D. The effect of combination was additive to synergistic (CI < 1.1) with AZ IC₂₀ ($p \leq 0.05$), while strong synergy was evident with AZ IC₄₀ ($p \leq 0.01$).

The effect of drug combinations was evaluated in tumor spheroid-formation assays, a method for determining the number of CSCs. We observed a significant decrease in number ($p \leq 0.01$) and size of spheroids following combination treatment compared to controls. Spheroid-formation efficiency decreased by two- and three fold on combination treatment with MX (IC₂₅) + AZ (IC₁₀) and MO (IC₂₅) + AZ (IC₁₀), respectively. (Figure 3E).

Combination Treatment Enhanced DNA Double-Strand Breaks and Apoptosis

Initially, we evaluated the effect of Dyrk1b inhibition alone, and found that Dyrk1b-i (IC₂₀) sensitized oral CSCs to apoptosis (Figure 4, A and B). Thereafter, to gain insight into the increased cell death evident on drug combination, we examined changes in γH2AX, the surrogate marker of DNA damage. As illustrated in Figure 4C, the percentage of γH2AX⁺ CSCs was higher in combination treatment than each drug alone ($p \leq 0.05$). DNA damage increased approximately 1.3- to 2.3-fold on combination. In the presence of DNA damage, apoptosis ensues if damage is beyond repair. Accordingly, we monitored the apoptotic response of CSCs by flow cytometry post-annexin V staining. Representative data for the apoptotic index are shown in Figure 4D. Treatment with MX and MO for 24 hours increased the proportion of apoptotic cells, and this increase was significantly high ($p \leq 0.05$) on combination with AZ. The increase was 1.4- to two fold higher on combination treatment. Further, apoptosis increased considerably with time (72 hours, data not shown). This indicates that drug combinations induced chemosensitivity in CSCs, elucidated in part by an increase in DNA double-strand breaks, resulting in increased apoptosis. These data are indirectly consistent with the synergistic effect seen in growth inhibition.

Combination Treatment Increased Mitochondrial Stress

ROS are essential mediators in myriad biological processes, playing a dual role in physiological functions where an increase in ROS is detrimental to cell survival. Therefore, a delicate balance between intracellular ROS levels and ROS-defence mechanisms is required for tumor development and progression. As combined treatment suppressed tumor-cell proliferation, we examined mitochondrial functioning by assessing parameters

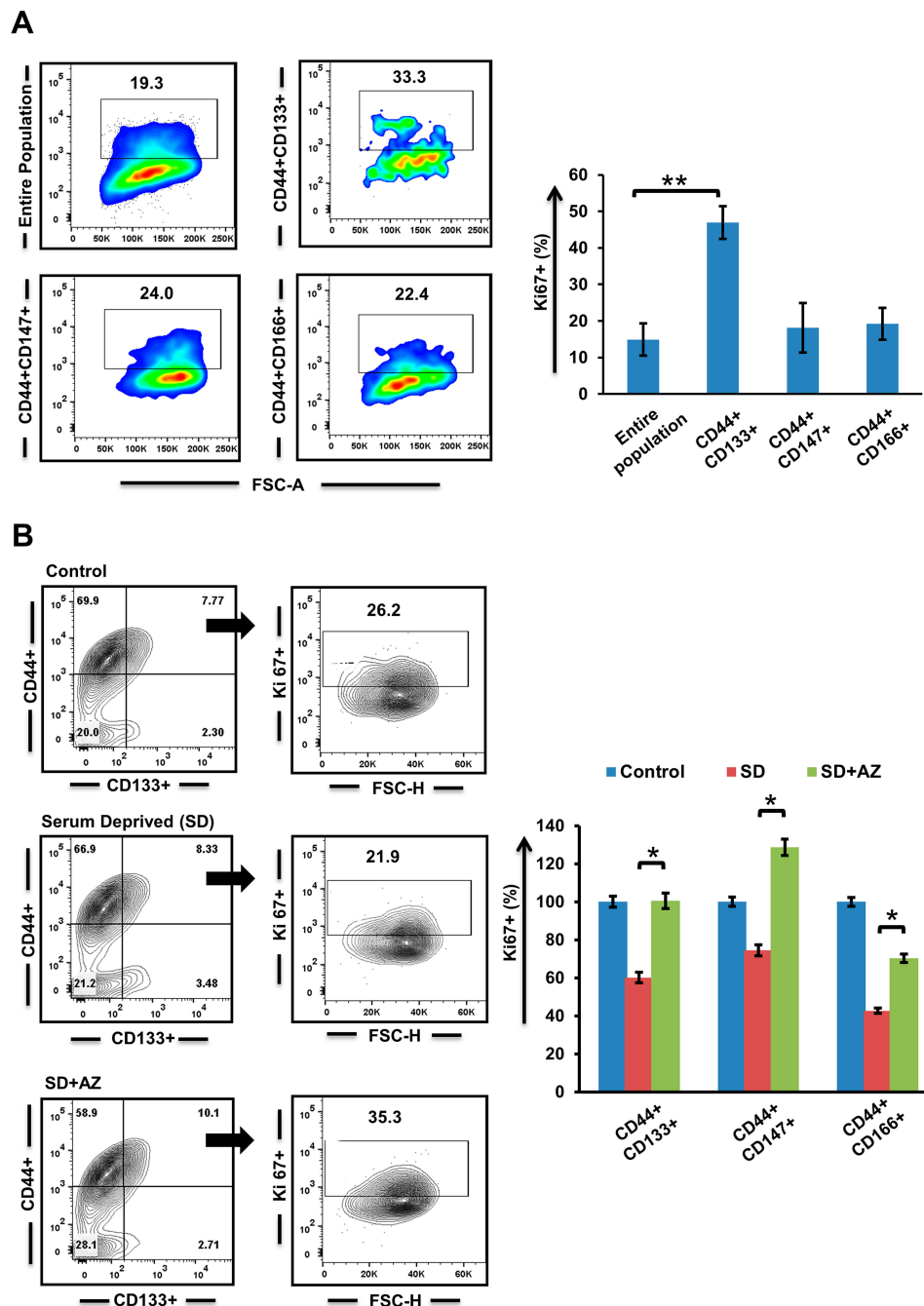


Figure 2 Evaluation of quiescence in cancer stem cells in (A) normal and (B) growth factor-deprived conditions post-Dyrk1b inhibition (n=4). * $p \leq 0.05$; ** $p \leq 0.01$.

impacting mitochondrial function. Combination of MX and MO with AZ showed reduced mitochondrial biogenesis compared to MX and MO alone, but the results were not significant ($p > 0.05$, Figure 5A). Increased mitochondrial stress in CSCs indicated by increased mitochondrial membrane depolarization ($p \leq 0.05$) was observed on combination treatment (Supplementary Figures 2A and 5B). We further demonstrated that drug combinations triggered

ROS production ($p \leq 0.05$) and decreased levels of reduced thiols ($p \leq 0.05$) with ThiolTracker violet staining (Supplementary Figure 2B, Figure 5, C and D). Thereupon, an increase in cytochrome C ($p \leq 0.01$) post-combination treatment was observed (Figure 5E). Collectively, these data shows that the combination approach enhanced mitochondrial stress and induced death in oral CSCs.

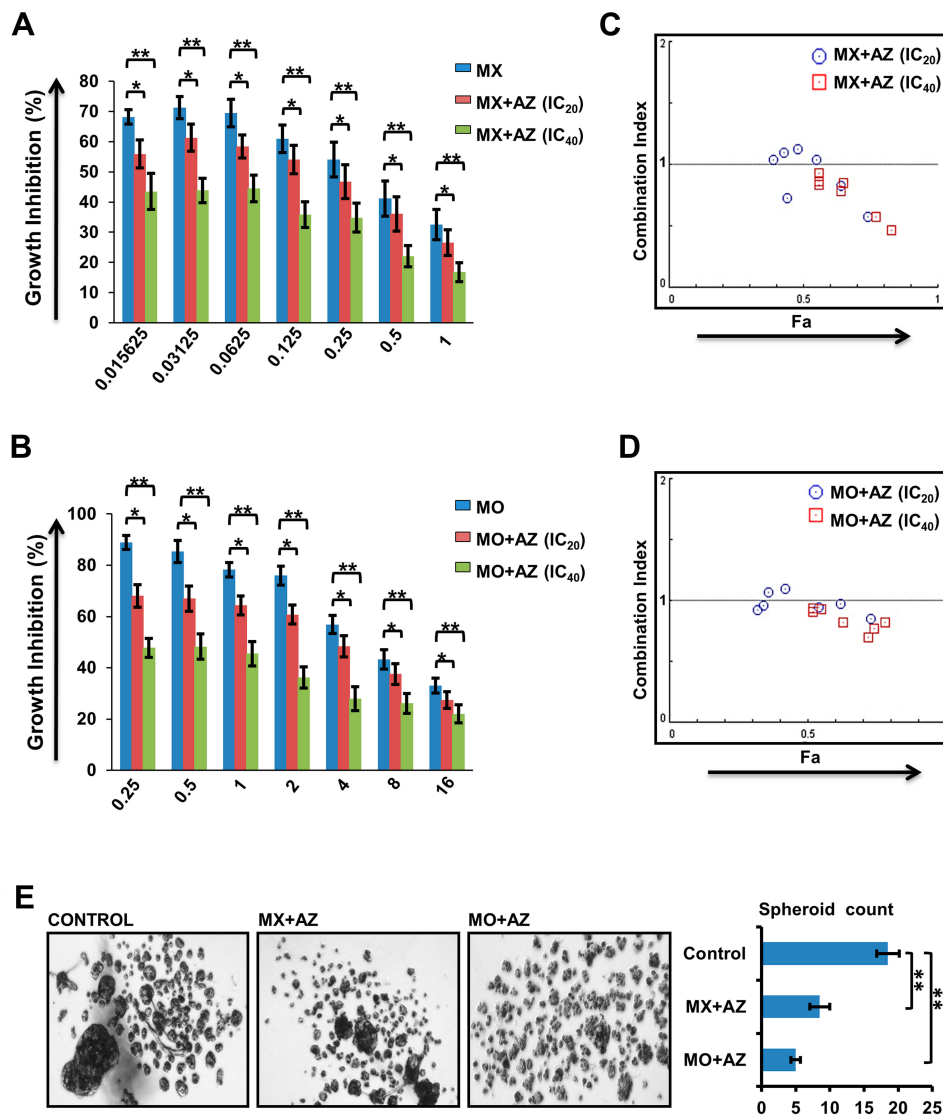


Figure 3 Dyrk1b inhibitors synergized with topoisomerase II and HDAC inhibitors (n=10). **(A)** Growth inhibition of primary oral squamous-cell carcinoma (OSCC) cells treated with mitoxantrone (MX) + AZ191 (AZ) and **(B)** mocetinosat (MO) + AZ. **(C)** Results of drug–drug interaction for MX-AZ and **(D)** MO-AZ evaluated using the combination index; values <1.0, 1.0–1.1, and >1.1 indicate synergy, additive effect, and antagonism respectively. **(E)** Spheroid-forming efficiency of OSCC cells on combination treatment. * $p \leq 0.05$; ** $p \leq 0.01$.

Efficient Targeting of Sox2⁺ Cancer Stem Cells

Accumulating evidence suggests SOX2 plays an indispensable role in mediating tumor progression and therapy resistance. As such, targeting Sox2⁺ cancer cells may potentially lead to better survival prospects. We evaluated DNA damage and cytochrome c levels in Sox2⁺ cancer cells post-combination treatment. Significant increases in γ H2AX expression and cytochrome c levels in Sox2⁺ cells ($p \leq 0.01$) compared to double-positive CSCs were observed (Figure 6, A and B; Supplementary Figure 3).

Discussion

CSCs possess tumor-initiating ability and are critical in the development of metastasis and recurrence. Their role in mediating drug resistance is one of the main reasons for therapy failure.^{25–27} The existence of CSCs in malignancies like colorectal cancer, prostate cancer, gliomas, pancreatic cancer, and breast and lung carcinoma^{28–31} have been associated with chemoresistance and poor prognosis. Through this study, we immunophenotypically characterized putative CSCs in OSCC based on the expression of known CSC-specific markers^{2–4,32} and the undifferentiated cell marker SOX2, and showed that

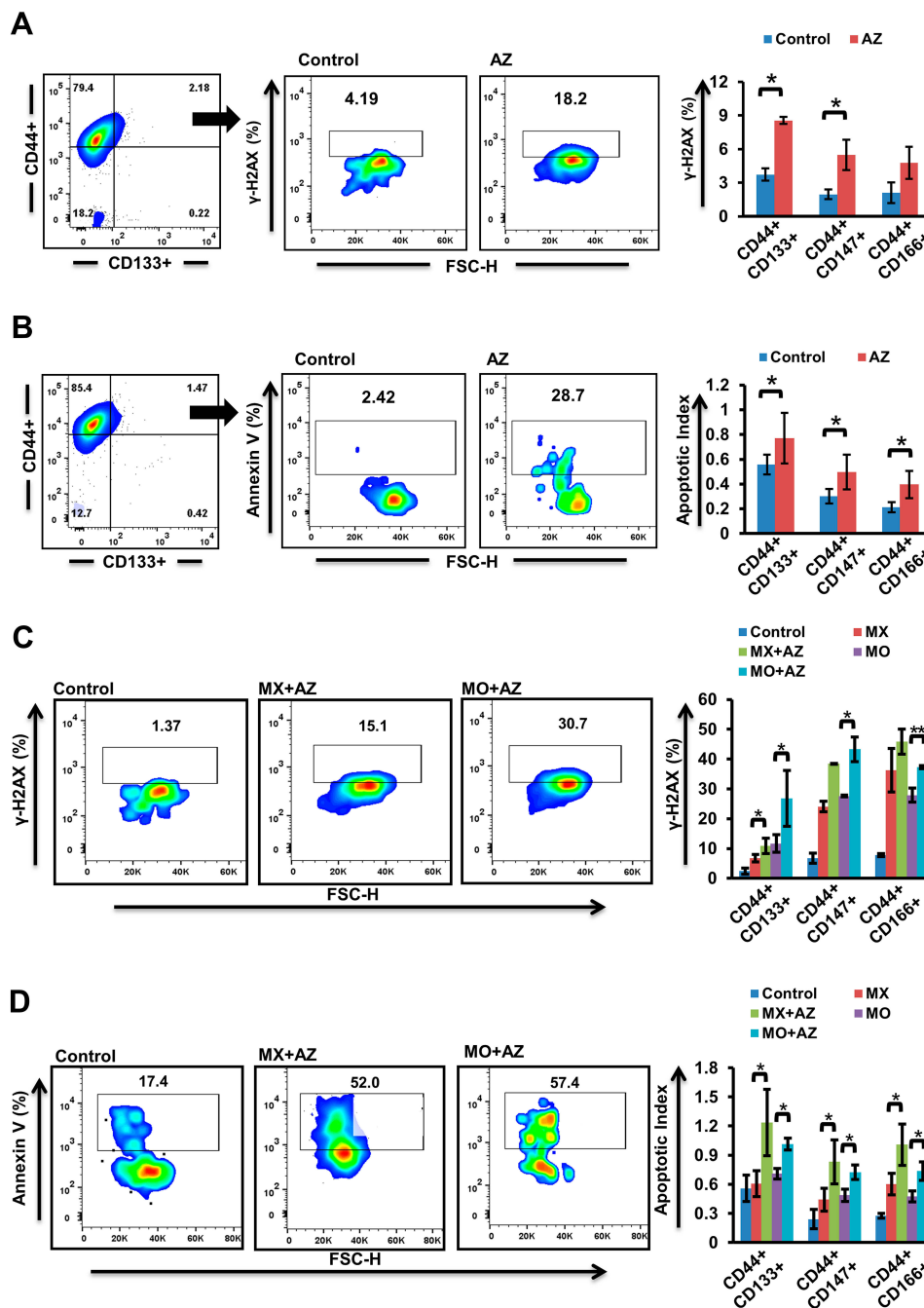


Figure 4 Effect of Dyrk1b inhibition and combination treatment (n=4). **(A)** Increase in γ -H2AX and **(B)** apoptosis in cancer stem cells (CSCs) 24 hours after Dyrk1b inhibition. **(C)** Changes seen in expression of γ -H2AX after 24 hours' treatment with mitoxantrone (MX), mocetinostat (MO), and combinations (MX+AZ191, MO+AZ191). **(D)** Analysis of apoptosis in CSCs treated with MX, MO, MX+AZ, and MO+AZ. The percentage of apoptotic cells was evaluated using annexin V staining, and the apoptotic index was determined. * $p \leq 0.05$ and ** $p \leq 0.01$.

inhibiting Dyrk1b in combination with known drugs is therapeutically relevant.

We selected multiple markers, as no individual marker can be used universally for identifying CSCs.^{5,32,33} Primary OSCC cells were positive for CD44, CD133, CD147, and CD166, though the percentage varied

slightly among cases. The expression of SOX2, a transcription factor that helps several stem cells in maintaining stemness and is usually upregulated in tumor cells with stem cell-like traits,^{4,34} was significantly high in double-positive CSCs. Among double-positive CSCs, CD44⁺CD133⁺ cells showed the highest

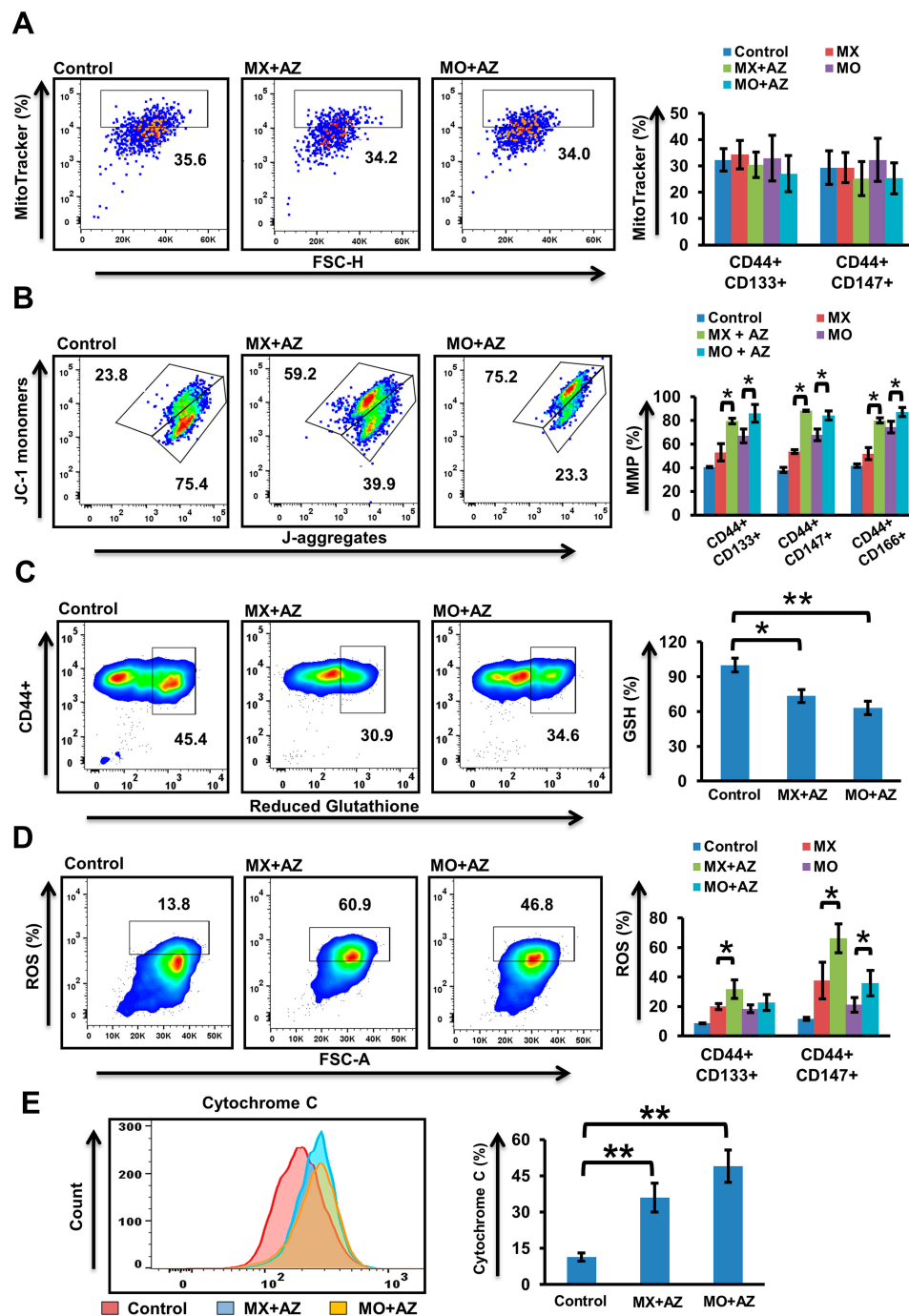


Figure 5 Quantification of mitochondrial stress (n=4). (A) Mitochondrial content in CSCs after 24 hours' treatment with mitoxantrone (MX), mocetinostat (MO), and combinations (MX+AZ191, MO+AZ191) using MitoTracker deep red. (B) Mitochondrial membrane potential in CSCs using JC1 dye was assessed after 24 hours' treatment with MX, MO, MX+AZ191, and MO+AZ191. JC1 ratio = $(\%green)/(\%green+\%red) \times 100$. (C) Intracellular glutathione levels determined using ThiolTracker violet in primary OSCC cells after 24 hours' combination treatment (MX+AZ191 and MO+AZ191). (D) ROS expression in CSCs using CellRox deep red following drug treatment. (E) Effect of combination treatment on cytochrome c levels. * $p \leq 0.05$; ** $p \leq 0.01$.

expression of SOX2 (29.1 ± 9.6). This further strengthened our approach of identifying an enriched population of oral CSCs, as CD133 is a known stem-cell marker and its expression usually associated with poor prognosis.^{35–39}

The major hurdle in cancer treatment is effective elimination of slowly dividing quiescent tumor cells, which show limited chemotherapeutic sensitivity, as majority of drugs are cell cycle-dependent. We found that in serum-deprived conditions, CSCs became more quiescent.

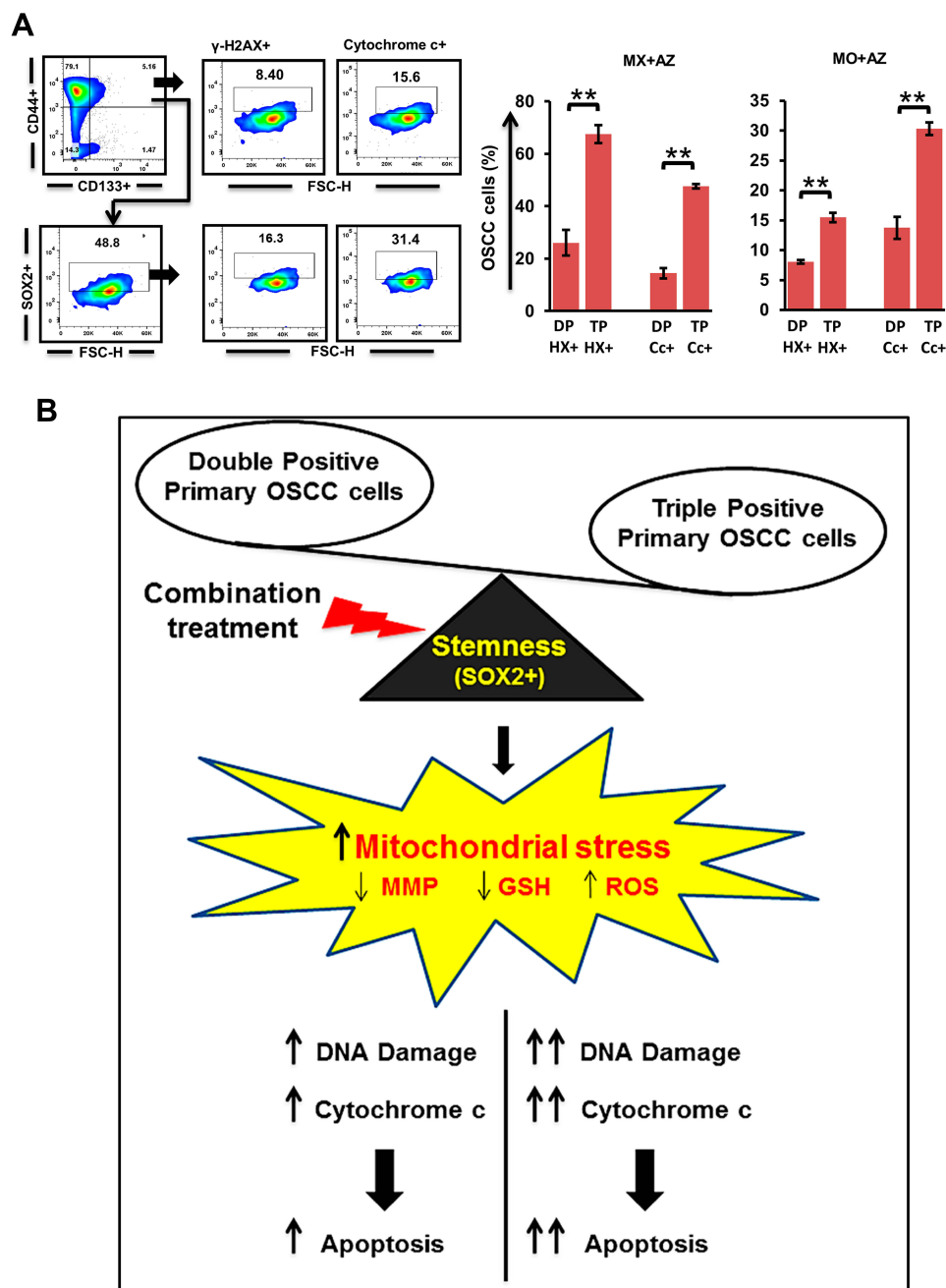


Figure 6 Quantification of DNA damage in Sox2⁺ cancer stem cells (CSCs; n=3). **(A)** γ H2AX and cytochrome c levels in triple-positive (CD44⁺CD133⁺Sox2⁺) cells compared to double-positive tumor cells. **(B)** Mechanism model of our combination approach to target oral CSCs. ****** $p \leq 0.01$.

Abbreviations: DP, double-positive (CD44⁺CD133⁺); TP, triple-positive (CD44⁺CD133⁺Sox2⁺); HX, γ H2AX; Cc-, Cytochrome c.

However, under similar conditions, treatment with Dyrk1b-i showed a significant decrease in quiescence ($p \leq 0.05$). This finding provided a compelling rationale for further investigation into the effect of combining Dyrk1b-i and chemotherapeutic drugs with different modus operandi. We observed that the combination of Dyrk1b-i with Topo-i and HDAC-i showed synergistic

activity ($p \leq 0.01$), and this effect was evident even at 16-fold lower IC₅₀. We studied non-constant drug-ratio combinations, and both MX and MO showed significant synergy in combination with AZ. Furthermore, drug combinations markedly increased DNA damage in CSCs ($p \leq 0.05$). This is an encouraging finding, as combination treatments aim to minimize therapeutic resistance. We

found that this combination approach was effective in targeting CSCs by disrupting mitochondrial function.

CSCs maintain ROS levels within limits as a function of the antioxidant system, of which the critical reducing agent is glutathione (GSH). Increased GSH has been implicated in chemotherapy resistance. Several studies have demonstrated that CSC survival depends largely on antioxidant machinery, and interference with their cellular antioxidant system is potentially detrimental.^{40,41} As such, an effective way to target CSCs is to synergize the cytotoxicity of ROS-producing chemotherapeutic drugs. Drugs used in the current study enhanced loss of mitochondrial membrane potential and modulated ROS production ($p \leq 0.05$). The combination of AZ with MX led to >50% increase in membrane depolarization, while its combination with MO resulted in approximately 28%, 24%, and 17% additional loss of mitochondrial membrane potential in CD44⁺CD133⁺, CD44⁺CD166⁺, and CD44⁺CD147⁺ cells, respectively. Addition of AZ also enhanced ROS production in CSCs. An increase of nearly 30% in ROS was found in CD44⁺CD133⁺ and 56% in CD44⁺CD147⁺ cells. The present study also evinced significant depletion in levels of GSH ($p \leq 0.05$) on combination treatment. Therefore, the underlying mechanism involved mitochondrial dysfunction and subsequent release of cytochrome c to cytosol, leading to apoptosis of oral CSCs. Altogether, our findings may thus be an avenue to treatment of oral cancer with combination therapy.

In conclusion, we report marker-based identification of CSC subpopulations and synergy of Dyrk1b inhibitors with topoisomerase II and HDAC inhibitors in primary OSCC. Through our drug-combination strategy, it is also possible to target quiescent CSCs. We showed that when Dyrk1b was inhibited, it allowed CSCs to acquire a proliferating cellular state, revealed by increased Ki67 expression. Importantly, apart from conferring proliferation, Dyrk1b inhibition simultaneously sensitized cancer cells to apoptosis. This means that its combination with Topo-i and HDAC-i manifested a dual effect, targeting both quiescent and proliferating tumor cells simultaneously.

With the ever-changing landscape of cancer, the development and trials of new drugs are growing rapidly. Combination studies provide a platform to identify drug candidates for a possible clinically relevant synergistic effect. The results of our study are vindication for additional exploration of drug combinations in the clinical setting to reduce the likelihood of developing tumor relapse. Future studies need to focus on identifying those targets for combination treatment that

minimize tumor drug resistance and simultaneously provide optimum therapeutic benefits.

Abbreviations

CSCs, cancer stem cells; OSCC, oral squamous cell carcinoma; Dyrk1b-i, Dyrk1b inhibitor; Topo-i, topoisomerase II inhibitor; HDAC-i, histone deacetylase inhibitor; pan-CK, pancytokeratin; MX, mitoxantrone; MO, mocetino-stat; AZ, AZ191; ROS, reactive oxygen species; GSH, glutathione.

Data Sharing Statement

The data for the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors declare that they have no competing interests in this work.

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