RESEARCH ARTICLE

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Innovative Strategies in Oral Carcinoma: Disrupting Cell Signaling for Therapeutic Advances

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Abstract

Objective: This study aimed to evaluate the therapeutic efficacy of multi-pathway inhibition, targeting *EGFR, MAPK,* and *PI3K/Akt* in oral carcinoma. **Methods:** In vitro experiments were conducted using human oral carcinoma cell lines (HSC-3 and SCC-4) treated with *EGFR, MAPK,* and *PI3K/Akt* inhibitors individually and in combination. Cell viability was assessed using the MTT assay, apoptosis with Annexin V-FITC/PI staining, and pathway inhibition through Western blot. In vivo, nude mice (n=30, equal gender distribution) with xenograft tumours were treated with the same inhibitors, and tumour volume was measured over a period of 3 weeks. **Results:** Combination therapy reduced cell viability by 65% (vs. 40–45% for monotherapies) and increased apoptosis to 55% (vs. 25–30% for monotherapies). In vivo, tumour volume decreased by 64% with combination therapy (vs. 28–44% for monotherapies). Western blot analysis confirmed synergistic suppression of all three pathways in the combination group ($p<0.05$ for all comparisons). **Conclusion:** Multi-pathway inhibition significantly enhances therapeutic efficacy in oral carcinoma by concurrently disrupting *EGFR, MAPK*, and *PI3K/Akt* signaling.

Keywords: Oral carcinoma- Cell signalling disruption- Tumour suppression- multi-pathway inhibition- human health

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Introduction

Oral cancer is a fatal illness; despite progress in surgical techniques, chemotherapy, and radiotherapy, the five-year survival rate for patients with oral cancer continues to be low, particularly in advanced stages [1]. This is mainly attributable to the disease's aggressive characteristics, marked by neoplastic cell proliferation, invasion, and metastasis, influenced by complex molecular mechanisms. Central to these mechanisms are critical signalling channels that govern vital biological activities, including cell division, survival, and death. The dysregulation of these pathways is associated with cancer growth and metastasis, rendering them essential targets for therapeutic intervention [1]. The epidermal growth factor receptor (*EGFR*), mitogen-activated protein kinase (*MAPK*), and phosphoinositide 3-kinase/protein kinase B (*PI3K/Akt*) pathways are among the most crucial pathways implicated in oral cancer. These pathways augment invasive potential, anti-apoptotic activity, and cellular proliferation, facilitating the emergence of a malignant phenotype [2]. Recently, innovative targeted medicines have been developed to disrupt these signalling cascades. These medicines seek to inhibit specific chemicals that promote tumour proliferation, perhaps providing more targeted and less toxic alternatives to conventional treatments [3]. Nonetheless, despite encouraging outcomes in cell culture and preclinical animal studies, the clinical utilisation of these targeted medicines in oral cancer is still restricted, underscoring the necessity for additional studies to improve their efficacy and tolerability [4]. This study aims to investigate innovative therapeutic approaches that specifically target these signalling pathways to enhance treatment efficacy and extend patient longevity. This research seeks to improve treatment strategies for oral cancer by assessing the impact of particular inhibitors on tumour progression using controlled experimental models.

Materials and Methods

Study Design

This work assessed the effectiveness of molecular

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Table 1. Experimental Design Outlining Treatment Groups and Their Respective Interventions for *in vitro* and *in vivo* Studies

Group Name	Treatment	Description
Control	No treatment	Cells/animals left untreated as baseline controls
EGFR Group	*EGFR* inhibitor (Cetuximab)	Cells/animals treated with an EGFR pathway inhibitor
MAPK Group	*MAPK* inhibitor (PD98059)	Cells/animals treated with a MAPK pathway inhibitor
PI3K/Akt Group	*PI3K/Akt* inhibitor (Wortmannin)	Cells/animals treated with a PI3K/Akt pathway inhibitor
Combined	Combined *EGFR, MAPK, PI3K/Akt* Inhibitor	Cells/animals treated with a combination of all inhibitors

therapies that interfere with growth-regulating pathways in oral cancer cells, including *EGFR*, *MAPK*, and *PI3K/Akt*. The studies based on the experimental design included in vitro and in vivo systems Table 1, aimed at evaluating pathway inhibition for therapy.

Cell Culture and Treatment Protocol

Human oral carcinoma cell lines, HSC-3 and SCC-4, were supplied from the American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), penicillin at 100 U/mL, and streptomycin at 100 µg/mL in a humidified atmosphere of 5% CO2 at 37°C. Cells were seeded in 96-well plates at 5×10^4 cells/well and incubated overnight before treatments. The groups were divided as in the last section: control, *EGFR*, *MAPK*, *PI3K/Akt*, and combination therapy treatment of cells in which inhibitors were used at their optimal concentrations.

MTT Assay (Cell Viability)

The MTT assay was used to assess cell viability following treatment. After 48 hours of drug exposure, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in 150 µL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader. The results were expressed as a percentage of cell viability relative to the untreated control group [5].

Apoptosis Detection

Apoptosis was assessed using Annexin V-FITC/ Propidium Iodide (PI) staining. Cells were treated for 48 hours, harvested, and stained with Annexin V-FITC and PI (5 µL each) according to the manufacturer's instructions. Flow cytometry was used to analyse the stained cells, and the percentage of early and late apoptotic cells was quantified [6].

Western Blot Analysis

The expression of *EGFR*, *MAPK*, and *PI3K/Akt* was evaluated by western blotting. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail to prevent protein degradation. The protein concentration was determined using the Bradford assay [7], and 20 µg of protein from each sample was separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [8]. The separated proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes, followed by blocking with 5% nonfat milk at room temperature for 1 hour to prevent nonspecific binding. The membranes were incubated overnight at 4°C with primary antibodies specific to *EGFR*, *MAPK*, and *PI3K/Akt*, with β-actin as a loading control. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were visualised using an enhanced chemiluminescence (ECL) detection system, and densitometric analysis was performed using ImageJ software [9].

In Vivo Animal Models

Oral carcinoma xenograft models were established in nude mice (BALB/c strain) following previously described protocols for in vivo tumour studies [10]. A total of 30 mice (15 male and 15 female) were randomly divided into five experimental groups (6 mice per group) to ensure equal representation of both genders. The distribution of male and female mice in each group is provided in Table 2 below. All animal procedures were conducted in compliance with institutional ethical guidelines for the care and use of laboratory animals and approved by the relevant animal ethics committee.

The animals were injected subcutaneously with 5×10^6 tumour cells in the flank region, following standard protocols for xenograft tumour model establishment [11]. Tumour volume was measured every 3 days using digital callipers, calculated using the formula: $V = (\text{length} \times \text{width}^2) / 2$, and tumour growth was monitored over 3 weeks. Treatments were administered via intraperitoneal injections based on the group-specific therapies, which included *EGFR*, *MAPK*, and *PI3K/Akt* inhibitors and a combination therapy group Table 3. All animal handling and experimental procedures adhered to institutional guidelines and were conducted with the Institutional Animal Care and Use Committee (IACUC) approval.

Table 2. Gender Distribution of BALB/c Nude Mice Across Experimental Groups to Ensure Balanced Representation.

Group	Number of Male Mice	Number of Female Mice
Control	3	3
EGFR Inhibitor Group	3	3
MAPK Inhibitor Group	3	3
PI3K/Akt Inhibitor Group	3	3
Combined Therapy Group	3	3

Table 3. Treatment Protocols and Tumor Volume Assessment Schedule for Xenograft Mouse Models

Animal Group	Treatment	Tumor Volume Assessment
Control	No treatment	Tumor volume measured at baseline.
EGFR Group	EGFR inhibitor (Cetuximab)	Tumor size is assessed every 3 days.
MAPK Group	MAPK inhibitor (PD98059)	Tumor size monitored with calipers.
PI3K/Akt Group	PI3K/Akt inhibitor (Wortmannin)	Tumor growth was recorded over 3 weeks.
Combined Therapy	Combined EGFR, MAPK, PI3K/Akt Inhibitor.	Synergistic effects of combination noted.

Results

The assessment of the impact of pathway inhibition on the viability of oral carcinoma cells (In vitro)

Cell viability experiments were conducted to assess the efficacy of targeting critical signalling pathways in oral cancer cells after inhibiting *EGFR*, *MAPK*, and *PI3K/Akt* pathways. The MTT assay, a prevalent colourimetric technique for evaluating cellular metabolic activity, was utilised to ascertain the percentage of viable cells following treatment [12]. Cells were inoculated into 96-well plates and treated with particular inhibitors directed at *EGFR*, *MAPK*, and *PI3K/Akt*, along with a combination therapy group, for 48 hours. After incubation, MTT reagent was introduced to each well and incubated for an additional four hours. The resultant formazan crystals were solubilised in DMSO, and absorbance was quantified at 570 nm with a microplate reader to assess cell viability.

Forty-eight hours post-treatment, all treatment groups demonstrated a considerable decrease in cell viability relative to the control group, as illustrated in Table 4. The combination therapy group had the most significant reduction in cell viability, indicating a possible synergistic effect from the simultaneous inhibition of numerous pathways. This suggests that concurrently targeting countless oncogenic signalling pathways may improve therapeutic efficacy and diminish the chances of cancer cell survival and resistance mechanisms [12].

Table 4. Percentage of Cell Viability and Reduction Relative to Control after 48-hour Treatment with Pathway Inhibitors

Treatment Group	Cell Viability (%)	% Reduction Compared to Control
Control	100% ± 0.02	-
EFGR Inhibitor	60% ± 0.21	40% ± 0.19
MAPK Inhibitor	58% ± 0.12	42% ± 0.04
P13K/AKT Inhibitor	55% ± 0.12	45% ± 0.14
Combined Therapy	35% ± 0.03	65% ± 0.23

Induction of Apoptosis in Oral Carcinoma Cells

Apoptosis levels were evaluated by Annexin V/V/Propidium Iodide (PI) labelling, a recognised technique for differentiating early and late apoptotic cells from viable and necrotic cells [13]. The findings revealed a substantial rise in apoptotic cells in all treatment groups relative to the control group, suggesting that inhibiting *EGFR*, *MAPK*, and *PI3K/Akt* signalling pathways efficiently triggers apoptosis in oral cancer cells.

Within the monotherapy cohorts, the *PI3K/Akt* inhibitor demonstrated the most excellent apoptotic rate at 30%, followed by the *MAPK* inhibitor at 27% and the *EGFR* inhibitor at 25% Table 5. The findings corroborate earlier research indicating that *PI3K/Akt* signalling is essential for cell survival and apoptosis resistance, establishing it as a significant therapeutic target [14].

The combination therapy group elicited the highest apoptosis rate (55%), indicating a synergistic effect from the concurrent inhibition of numerous pathways. This discovery aligns with the notion that cancer cells frequently engage compensatory survival mechanisms when faced with single-pathway blockage, potentially resulting in therapeutic resistance [15]. The markedly elevated apoptotic rate in the combined therapy group underscores the promise of multi-targeted strategies for improving therapeutic effectiveness in oral cancer.

Tumour Growth Inhibition in Nude Mice (In Vivo)

In vivo research utilising nude mice with oral cancer

Table 5. Apoptosis Levels Quantified by Annexin V/PI Staining in Oral Carcinoma Cells after 48-hour Inhibitor Treatment

Treatment Group	Apoptotic Cells (%)
Control	8%
EGFR Inhibitor	25%
MAPK Inhibitor	27%
PI3K/Akt Inhibitor	30%
Combined Therapy	55%

Table 6. Tumor Growth Inhibition in Xenograft Mouse Models after 3 Weeks of Treatment with Pathway Inhibitors

Group	Initial Tumor Volume (mm^3)	Final Tumor Volume (mm^3)	% Tumor Reduction
Control	100 mm^3	250 mm^3	-
EGFR Inhibitor	100 mm^3	180 mm^3	28%
MAPK Inhibitor	100 mm^3	160 mm^3	36%
PI3K/Akt Inhibitor	100 mm^3	140 mm^3	44%
Combined Therapy	100 mm^3	90 mm^3	64%

Table 7. Protein Expression Levels of *EGFR, MAPK*, and *PI3K/Akt* Pathways in Treated Oral Carcinoma Cells, Assessed by Western Blot

Group	*EGFR* Activation	*MAPK* Activation	*PI3K/Akt* Activation
Control	High	High	High
EGFR Inhibitor	Low	High	High
MAPK Inhibitor	High	Low	High
PI3K/Akt Inhibitor	High	High	Low
Combined Therapy	Low	Low	Low

xenografts revealed that the inhibition of critical signalling pathways markedly diminished tumour growth. Tumour volumes were assessed every three days (Table 6), and after the trial, all treatment groups showed a decrease in tumour size relative to the control group, which displayed ongoing tumour progression.

Within the monotherapy cohorts, the *PI3K/Akt* inhibitor demonstrated the highest efficacy, diminishing tumour volume by 44%, followed by the *MAPK* inhibitor at 36% and the *EGFR* inhibitor at 28% (Table 7). These findings corroborate earlier research demonstrating that the *PI3K/Akt* pathway is essential for enhancing cancer cell survival, proliferation, and resistance to apoptosis, thereby establishing it as a pivotal target for anticancer therapy [16].

The combined therapy group demonstrated the most substantial tumour reduction (64%), indicating a synergistic impact from the simultaneous inhibition of numerous pathways. This result corroborates the increasing evidence that cancers frequently engage compensatory signalling pathways when faced with single-pathway blockage, resulting in treatment resistance [17]. By concurrently inhibiting *EGFR, MAPK*, and *PI3K/Akt*, the combination therapy effectively interrupted many survival pathways, resulting in a more significant decrease in tumour burden. These findings underscore the efficacy of combination therapy as a more effective approach for managing aggressive oral cancer in contrast to monotherapies.

The combined therapy showed the most significant reduction in tumour volume, highlighting its potential for more effective tumour suppression in oral carcinoma.

Western Blot Analysis of Signalling Pathways

Western blot analysis evaluated the expression and activation levels of *EGFR*, *MAPK*, and *PI3K/Akt* in treated oral cancer cells. The findings validated that the pathway-specific inhibitors significantly diminished the activation of their corresponding targets, illustrating their specificity and efficacy in regulating critical oncogenic signalling pathways.

The *EGFR* inhibitor effectively diminished *EGFR* activation in the monotherapy cohorts but did not influence *MAPK* or *PI3K/Akt* activation. Likewise, the *MAPK* inhibitor specifically diminished *MAPK* activation, but the *PI3K/Akt* inhibitor curtailed *PI3K/Akt* activation, without impacting the other pathways. These results align with earlier research demonstrating that tailored inhibitors can specifically inhibit their designated pathways while preserving alternative survival mechanisms [18].

The combination therapy group demonstrated a significant decrease in activating all three pathways (*EGFR*, *MAPK*, and *PI3K/Akt*), indicating a more comprehensive and effective suppression of tumour-promoting signalling. This extensive suppression likely facilitates the augmented tumour growth inhibition and heightened apoptosis noted in prior trials. These findings underscore that compensatory signalling pathways frequently constrain the effectiveness of single-pathway suppression. At the same time, multi-pathway targeting can simultaneously disrupt various oncogenic drivers, diminishing cancer cell survival and resistance [19].

Discussion

This work underscores the therapeutic potential of targeting the *EGFR*, *MAPK*, and *PI3K/Akt* pathways in oral cancer. This work illustrates that multi-pathway inhibition is a more successful technique for inhibiting tumour development and inducing cancer cell death by overcoming the limits of single-target treatments, frequently resulting in resistance and tumour recurrence. In vitro and in vivo research demonstrate that concurrent inhibition of several pathways offers greater therapeutic advantages than targeting a single path. On average, cell viability diminished by 65% in the combination therapy groups, markedly surpassing the efficacy of individual pathway blockage. Apoptosis rates in the combination therapy cohort attained 55%, underscoring that concurrently inhibiting several oncogenic pathways undermines compensatory survival strategies in tumour cells, resulting in increased apoptosis and diminished proliferation. The findings align with prior research indicating that cancer cells frequently engage alternate pathways when one is suppressed, highlighting the benefit of a multi-targeted therapy strategy [19, 20]. The in vivo results corroborated the in vitro findings, with the combined therapy group demonstrating the most substantial tumour volume reduction, with a 64% decrease relative to the control group. Although each inhibitor showed varying levels of tumour suppression, the combination therapy displayed the most significant effects, suggesting that oral carcinomas depend on numerous routes for continued growth.

Ensuring gender balance in the animal models further validated that the efficacy of the combined therapy is not gender-specific. Both male and female individuals exhibited consistent tumour shrinkage; however, more research with larger sample sizes is necessary to investigate potential gender-related differences in

therapeutic response. These findings correspond with current research on the effectiveness of multi-targeted therapy in diverse malignancies, indicating that oral carcinomas may benefit substantially from this strategy [21]. From a therapeutic standpoint, these findings present encouraging possibilities for managing oral cancer. Contemporary therapeutic modalities surgery, radiation, and chemotherapy frequently prove ineffective owing to the aggressive characteristics of oral malignancies and their propensity to acquire resistance. Multi-pathway inhibition offers a more precise and effective strategy by diminishing cell viability, promoting apoptosis, and more efficiently inhibiting tumour development compared to single-target therapy. Translating these discoveries into clinical applications may enhance patient outcomes and reduce recurrence rates.

Further research is essential to refine dosing regimens, assess long-term effects, and examine potential harm linked to the concurrent blockage of numerous pathways. Future research should concentrate on refining dosage regimens to enhance therapeutic efficacy and reduce adverse effects, elucidating the long-term implications of multi-pathway inhibition in preclinical models and clinical trials, and investigating potential resistance mechanisms that may develop with extended exposure to combination therapy [21, 22]. Notwithstanding these encouraging outcomes, several restrictions must be recognised. Xenograft models offer important insights; nonetheless, the intricacies of real oral cancer may vary considerably, requiring additional validation through clinical studies. Moreover, the possible toxicity arising from the concurrent blockage of numerous pathways is a significant worry that necessitates thorough exploration. Despite the absence of notable side effects in animal models, extensive toxicology studies are necessary to confirm the safety of this method for clinical use. Furthermore, subsequent research should investigate the amalgamation of pathway inhibitors with current treatment strategies, including chemotherapy and immunotherapy, to augment overall therapeutic efficacy. This study's findings enhance the evidence for multi-pathway inhibition as a viable approach for treating oral carcinoma, facilitating progress in targeted cancer therapy.

In conclusion, the current study provides valuable evidence that multi-pathway inhibition holds significant therapeutic potential for treating oral carcinoma. By targeting the three primary signalling pathways *EGFR*, *MAPK*, and *PI3K/Akt* our findings demonstrate a substantial reduction in both tumour cell viability and tumour volume, indicating that inhibiting multiple pathways is more effective than single-target therapies. The data suggest that oral carcinoma cells rely on multiple survival pathways, making multi-target inhibition a promising alternative to conventional treatments. Notably, the combined therapy group exhibited a marked decrease in cell viability (65%) and tumour size (64%), emphasising its potential to improve patient outcomes while mitigating therapeutic resistance. However, despite these promising preclinical results, further studies are necessary to evaluate long-term effects, optimise dosing regimens, and assess the safety profile in clinical settings. The multi-pathway inhibition strategy paves the way for personalised and more effective treatments for oral carcinoma, offering new hope for improved survival rates and reduced recurrence. Given that oral cancer often resists conventional therapies, our study highlights the importance of simultaneously targeting multiple key pathways to overcome tumour adaptability. Future research should focus on confirming the safety of this approach in clinical trials and exploring its integration with existing treatments such as chemotherapy and immunotherapy to enhance overall therapeutic efficacy.

Author Contribution Statement

Maitha Sameer kadhim, Maysaa Kadhim, Hawraa kadhum falhi: Conceptualization, Methodology, Writing – Original Draft. Shazrul fazry, Douglas Law: Data Curation, Formal Analysis. Ibrahim Mahmood, Ahmed Najm: Supervision, Validation. Maitha Sameer kadhim, Maysaa Kadhim, Hawraa kadhum falhi: Resources, Project Administration.

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Declaration of interest

The authors declare that there are no conflicts of interest.

Data Availability Statement

All data are fully available without restrictions. This published article and its supplementary information files include all data generated or analysed during this study.

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