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AVALIAÇÃO DOS EFEITOS DOS COMPLEXOS RuMTNZ 7 E RuMTNZ 9 SOBRE A PROLIFERAÇÃO E A MOTILIDADE DE CÉLULAS DE CARCINOMA ESPINOCELULAR FaDu: UMA ABORDAGEM *IN VITRO*

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Trabalho de Conclusão de Curso apresentado ao curso de Bacharelado em Ciências Biológicas, da Universidade Federal de Alfenas, como requisito para obtenção do título de bacharel em Ciências Biológicas.

Orientador: Prof. Dr. Angel Mauricio Castro Gamero

Co-orientador: Me. João Marcos Oliveira da Silva

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O Presidente da banca examinadora abaixo assina a aprovação do Trabalho de conclusão de curso apresentada como parte dos requisitos para obtenção do título de Bacharel em Ciências Biológicas pela Universidade Federal de Alfenas.

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DEDICATÓRIA

Àqueles que iluminaram meu caminho. Madame du Châtelet dizia que *'é preciso ousar pensar'* — e eu só ousei porque vocês sustentaram meus passos.

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“O começo de todas as ciências é o espanto de as coisas serem o que são.” Aristóteles

RESUMO

O carcinoma espinocelular de cabeça e pescoço HPV-negativo (CEC-CP) permanece como uma das malignidades mais desafiadoras em oncologia, caracterizado por forte heterogeneidade intracelular e genética, rápido avanço clínico e resistência persistente à cisplatina. Diante dessas limitações, complexos metálicos emergem como alternativas capazes de remodelar o cenário terapêutico. Entre eles, compostos de rutênio(II) conjugados ao metronidazol, RuMTNZ7 e RuMTNZ9, têm demonstrado citotoxicidade e modulação do ciclo celular em outros tumores, mas seus efeitos no CEC-CP permanecem inexplorados. Neste estudo, utilizamos esferoides derivados da linhagem celular FaDu para investigar, em profundidade, a ação de RuMTNZ7 e RuMTNZ9 sobre processos fundamentais da progressão tumoral tridimensional, avaliando proliferação, por meio do ensaio de crescimento de colônias em ágar semissólido, migração, pelo ensaio tridimensional, e invasão celular, pelo modelo 2D em insert. Ambos os complexos reduziram de forma contundente a formação de colônias, evidenciando uma atividade antiproliferativa vigorosa. Na migração, observou-se uma resposta marcadamente dependente da dose: concentrações equivalentes à IC_{50} aboliram a motilidade celular, enquanto em doses menores preservaram o deslocamento, indicando possível manutenção ou seleção de subpopulações altamente migratórias. Em contraste, nenhum dos compostos alterou a invasão celular, revelando uma dissociação funcional clara entre migração e invasão. Em conjunto, esses achados demonstram que RuMTNZ7 e RuMTNZ9 exercem efeitos seletivos sobre eixos críticos da biologia tumoral, suprimindo proliferação e migração sem afetar a capacidade invasiva. Esses resultados consolidam o potencial desses complexos de rutênio como candidatos inovadores em metalofarmacologia e reforçam a urgência de estudos futuros voltados a mecanismos moleculares e estratégias terapêuticas combinatórias, com foco no comportamento tumoral tridimensional.

Palavras-chave: Carcinoma espinocelular; rutênio; metalofármacos; RuMTNZ

ABSTRACT

HPV-negative head and neck squamous cell carcinoma (HNSCC) remains one of the most challenging malignancies in oncology, characterized by pronounced intracellular and genetic heterogeneity, rapid clinical progression, and persistent resistance to cisplatin. In light of these limitations, metal-based complexes have emerged as promising alternatives capable of reshaping the therapeutic landscape. Among them, ruthenium(II) complexes conjugated to metronidazole, RuMTNZ7 and RuMTNZ9, have demonstrated cytotoxicity and cell-cycle modulation in other tumor models; however, their effects in HNSCC remain unexplored.In this study, we employed spheroids derived from the FaDu cell line to investigate, in depth, the activity of RuMTNZ7 and RuMTNZ9 on key processes governing three-dimensional tumor progression, assessing proliferation through a soft agar colony formation assay, migration using a 3D migration assay, and cell invasion using a 2D insert-based model. Both complexes markedly reduced colony formation, demonstrating robust antiproliferative activity. Regarding migration, a clearly dose-dependent response was observed: concentrations corresponding to the IC_{50} abolished cellular motility, whereas lower doses preserved displacement, suggesting potential maintenance or selection of highly migratory subpopulations.In contrast, neither compound altered cell invasion, revealing a clear functional dissociation between migration and invasion. Collectively, these findings show that RuMTNZ7 and RuMTNZ9 exert selective effects on critical axes of tumor biology, suppressing proliferation and migration without affecting invasive capacity. These results highlight the potential of these ruthenium complexes as innovative candidates in metallopharmacology and underscore the need for future studies focused on molecular mechanisms and combinatorial therapeutic strategies, with particular emphasis on tridimensional tumor behavior.

Key-words: Squamous cell carcinoma; ruthenium; metallodrugs; RuMTNZ

LISTA DE SIGLAS:

2D - Bidimensional

3D - Tridimensional
CEC - Carcinoma espinocelular (Squamous Cell Carcinoma)

CEC-CP - Carcinoma espinocelular de cabeça e pescoço (*Head and Neck Squamous Cell Carcinoma*)

CDDP- Cisplatina (Cisplatin)

DMSO - Dimetil Sulfóxido (Dimethyl Sulfoxide)

HNSCC - *Head and Neck Squamous Cell Carcinoma*

HPV - Papiloma vírus humano (Human papillomavirus)

MEC- Matriz extracelular (extracellular matrix)

NCI- Instituto Nacional do câncer (National Cancer Institute)

RuMTNZ - Rutênio (II) com Metronidazol

SUMÁRIO

1. INTRODUÇÃO

De acordo com o *National Cancer Institute* (NCI), o câncer não constitui uma entidade única, mas um grupo complexo de enfermidades biologicamente distintas que podem surgir em praticamente qualquer tecido ou órgão do corpo humano. Nesse contexto heterogêneo, o câncer de pele não melanoma destaca-se como a malignidade mais frequentemente diagnosticada mundialmente, acometendo aproximadamente um milhão de pessoas por ano (DESAI et al., 2023). Essa elevada incidência reflete não apenas a ampla exposição da população a fatores ambientais de risco, mas também a necessidade contínua de estratégias terapêuticas mais eficazes e seletivas.

Dentro desse grupo de neoplasias, observa-se um aumento expressivo dos carcinomas espinocelulares (CEC) de cabeça e pescoço, que se originam do epitélio que reveste a mucosa da cavidade oral, faringe e laringe. Esse crescimento, estimado em cerca de 30% entre indivíduos jovens, tem sido amplamente associado a mudanças nos padrões de comportamento, sobretudo ao aumento do tabagismo e à maior exposição ao papilomavírus humano (HPV) (DESAI et al., 2023). Além desses determinantes, a exposição solar prolongada aos raios ultravioleta configura um importante fator de risco, especialmente entre pessoas de pele clara, contribuindo para o acometimento predominante de regiões fotoexpostas, como cabeça e pescoço (LI Z et al., 2025). Clinicamente, o CEC manifesta-se como lesões ulceradas ou verrucosas de cicatrização lenta, podendo apresentar dor e episódios ocasionais de sangramento, e seu tratamento envolve cirurgia, radioterapia ou sua combinação com quimioterapia (DESAI et al., 2023).

Entre os subtipos dessa doença, os carcinomas espinocelulares de hipofaringe apresentam prognóstico particularmente desfavorável. Frequentemente diagnosticados em estágios localmente avançados, eles já se encontram associados à infiltração regional e à presença de metástases em linfonodos cervicais no momento do diagnóstico (WYCLIFFE et al., 2007). A baixa taxa de sobrevida está diretamente relacionada à elevada capacidade metastática, um processo considerado uma das marcas registradas do câncer. A metástase decorre da perda de adesão célula–célula e da quebra da integridade epitelial, desencadeando um programa de migração e invasão essencial para a disseminação tumoral (HANAHAN & WEINBERG, 2011). Durante esse processo, células epiteliais adquirem características mesenquimais, passando a exibir plasticidade morfológica, aumento da motilidade, resistência

à apoptose e habilidade de remodelar a matriz extracelular, favorecendo a progressão e a agressividade tumoral (DONGRE & WEINBERG, 2019).

A busca por intervenções capazes de interromper tais processos motivou, historicamente, o desenvolvimento de metalofármacos na oncologia. A descoberta da atividade antitumoral da cisplatina inaugurou uma era de intensa investigação envolvendo compostos metálicos, resultando na síntese de múltiplos derivados de platina, dos quais apenas alguns avançaram para ensaios clínicos (LUCACIU et al., 2022). Embora eficazes, agentes baseados em platina enfrentam limitações significativas, como baixa seletividade e toxicidade sistêmica em células normais, o que impulsionou a busca por alternativas mais seguras e seletivas. Nesse cenário, o rutênio (Ru), um metal de transição com propriedades químicas versáteis, emergiu como candidato promissor na nova geração de metalofármacos (LEE et al., 2020).

Com reconhecida atividade antitumoral e menor toxicidade sistêmica, complexos de rutênio vêm sendo amplamente estudados. Suas vantagens incluem estabilidade em múltiplos estados de oxidação, baixa cinética de troca e similaridade química com o ferro, características que não apenas conferem maior versatilidade estrutural, como também permitem o desenvolvimento de pró-fármacos ativáveis em ambiente tumoral reduzido (SONKAR et al., 2021). Entre as formas mais estudadas, os complexos de rutênio nos estados II e III ocupam posição central como potenciais sucessores aos compostos de platina. Sua geometria octaédrica, distinta da estrutura quadrado-plana típica dos complexos de platina, possibilita modos de interação alternativos com o DNA, interferindo nos processos de replicação e transcrição e promovendo apoptose, inclusive por mecanismos lisossomais (SKOCZYNSKA et al., 2023).

Dentro desse grupo, uma abordagem particularmente promissora envolve a conjugação de rutênio(II) ao metronidazol (RuMTNZ). De acordo com Cândido e colaboradores (2022), esse tipo de complexo demonstrou capacidade de interagir com o DNA e bloquear a progressão do ciclo celular em modelo experimental de câncer de mama. Assim, a exploração de complexos RuMTNZ em diferentes contextos tumorais configura uma estratégia inovadora, capaz de expandir o repertório terapêutico disponível e enfrentar limitações associadas a terapias convencionais.

À luz desses avanços, torna-se evidente que os carcinomas espinocelulares de cabeça e pescoço, especialmente aqueles HPV-negativos e localmente avançados, continuam a demandar agentes terapêuticos mais eficazes e seletivos. Considerando o potencial singular dos complexos de rutênio, particularmente conjugado ao metronidazol, é crucial aprofundar a

compreensão de seus efeitos biológicos em modelos representativos da doença. Assim, investigar como os complexos RuMTNZ modulam processos fundamentais da progressão tumoral, como proliferação, migração e invasão, representa um passo decisivo para determinar sua aplicabilidade terapêutica e consolidá-los como candidatos de destaque na próxima geração de metalofármacos.

2. JUSTIFICATIVA

O carcinoma espinocelular (CEC) representa uma das neoplasias malignas de maior incidência mundial e caracteriza-se por marcada heterogeneidade histopatológica, elevada plasticidade biológica e recorrência local frequente. No âmbito específico dos carcinomas espinocelulares de cabeça e pescoço (CEC-CP), esse grupo tumoral constitui o sétimo tipo de câncer mais diagnosticado globalmente, acometendo estruturas revestidas por epitélio escamoso, incluindo cavidade oral, orofaringe, nasofaringe, hipofaringe, lábios, cavidade nasal, seios paranasais e glândulas salivares (BARSOUK et al., 2023). As estratégias terapêuticas atualmente utilizadas, predominantemente ressecção cirúrgica seguida de radioterapia, isolada ou combinada à quimioterapia, têm permitido avanços graduais no manejo clínico (JOHNSON et al., 2020). No entanto, o CEC-CP continua a apresentar comportamento altamente agressivo, refletido em sua grande capacidade de invasão local, rápida disseminação regional e resistência recorrente aos quimioterápicos convencionais.

Entre as terapias disponíveis, a cisplatina (CDDP) permanece como um dos pilares do tratamento sistêmico, devido à sua capacidade de induzir danos ao DNA e desencadear morte celular. Apesar de amplamente empregada, a CDDP apresenta limitações substanciais, incluindo baixa seletividade, toxicidade sistêmica acentuada e desenvolvimento progressivo de resistência adquirida (ELMORSY et al., 2024). Tais limitações comprometem tanto a eficácia terapêutica quanto a qualidade de vida dos pacientes, tornando evidente a urgência por alternativas farmacológicas mais seletivas, menos tóxicas e capazes de superar a refratariedade frequentemente observada nos CEC-CP.

Nesse cenário, os complexos metálicos de rutênio emergem como candidatos particularmente promissores. O rutênio distingue-se por apresentar múltiplos estados de oxidação estáveis, baixa toxicidade sistêmica e maior seletividade para células tumorais, além de propriedades estruturais que favorecem interações moleculares específicas. Essas características ampliam a possibilidade de desenvolver moléculas com mecanismos de ação diferenciados, seja pela interação direta com o DNA, seja pela modulação de organelas essenciais, como lisossomos e mitocôndrias, resultando em morte celular mais controlada e seletiva (SKOCZYNSKA et al., 2023).

Entre os compostos derivados de rutênio, destaca-se o complexo de rutênio(II) conjugado ao metronidazol, que demonstrou resultados promissores ao induzir apoptose e bloquear a progressão do ciclo celular em modelo experimental de câncer de mama, conforme

relatado por Cândido et al. (2022). Apesar desses avanços, permanece uma lacuna significativa no conhecimento: a aplicação desses complexos em modelos tumorais distintos, especialmente em tumores altamente agressivos e pouco responsivos à quimioterapia convencional, como os carcinomas espinocelulares de cabeça e pescoço.

Assim, o presente trabalho assume relevância científica ao explorar, de forma inédita, o papel biológico de complexos de rutênio associados ao metronidazol (RuMTNZ) frente ao CEC-CP. As moléculas utilizadas foram gentilmente fornecidas por meio de colaboração com o professor Dr. Antônio Carlos Doriguetto (UNIFAL-MG), assegurando a qualidade e a originalidade estrutural dos compostos.

No âmbito do nosso grupo de pesquisa, estudos preliminares indicaram que os complexos RuMTNZ exibem citotoxicidade significativa em modelos 2D e 3D da linhagem celular FaDu, além de apresentarem comportamento antagônico quando combinados à CDDP (ANEXO 01). Esses achados reforçam a importância de compreender, de forma detalhada, os efeitos dos complexos em monoterapia, particularmente no que se refere a processos essenciais da progressão tumoral, como motilidade celular, formação de colônias e plasticidade proliferativa.

Dessa forma, investigar os efeitos isolados dos complexos RuMTNZ sobre eventos relacionados à migração, invasão e expansão clonal torna-se não apenas justificável, mas crucial. Tal investigação poderá elucidar mecanismos biológicos até então pouco explorados, identificar potenciais vantagens terapêuticas desses compostos e consolidar o rutênio como um eixo estratégico para o desenvolvimento de uma nova geração de metalofármacos aplicáveis ao tratamento do carcinoma espinocelular de cabeça e pescoço.

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3 OBJETIVOS

3.1 OBJETIVOS GERAIS

Avaliar, em modelo *in vitro*, o efeito de RuMTNZ7 e RuMTNZ9 sobre a proliferação celular e na motilidade celular.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos de RuMTNZ7 e RuMTNZ9 sobre a proliferação celular da linhagem CEC FaDu;
- Estudar os efeitos de RuMTNZ7 e RuMTNZ9 sobre a migração celular 3D em esferóides derivados da linhagem FaDu;
- Investigar os efeitos de RuMTNZ7 e RuMTNZ9 sobre a capacidade de invasão celular, na linhagem FaDu.

4 DESENVOLVIMENTO

RUTHENIUM(II)–METRONIDAZOLE COMPLEXES DECREASE PROLIFERATION AND CELL MOTILITY IN HYPOPHARYNGEAL SQUAMOUS CELL CARCINOMA (FADU)

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ABSTRACT

HPV-negative head and neck squamous cell carcinoma (HNSCC) remains one of the most challenging malignancies in oncology, defined by pronounced heterogeneity, rapid clinical progression, and persistent resistance to cisplatin. Given these limitations, metal-based complexes have emerged as promising alternatives capable of reshaping the therapeutic landscape. Among them, ruthenium(II) complexes conjugated to metronidazole, RuMTNZ7 and RuMTNZ9, have demonstrated cytotoxic and cell-cycle-modulating properties in other tumor models; however, their effects in HNSCC remain largely unexplored. In this study, we used spheroids derived from the FaDu cell line to deeply investigate the activity of RuMTNZ7 and RuMTNZ9 on fundamental processes of tumor progression, assessing proliferation, through the semisolid agar colony-formation assay, migration, through the three-dimensional migration assay, and invasion, through the Boyden chamber assay. Both complexes markedly reduced colony formation, demonstrating a strong antiproliferative effect. In the migration assay, we observed a clear dose-dependent response: concentrations equivalent to the IC_{50} abolished cellular motility, whereas lower doses preserved movement, suggesting the possible maintenance or selection of highly migratory subpopulations. In contrast, neither compound altered invasion in the boyden chamber assay (3D), revealing a clear functional dissociation between migration and invasion. Taken together, these findings show that RuMTNZ7 and RuMTNZ9 exert selective effects on critical axes of tumor biology, suppressing proliferation and migration without affecting invasive capacity. These results consolidate the potential of these ruthenium complexes as innovative candidates in metallodrug development and reinforce the need for future studies focused on molecular mechanisms and combinatorial therapeutic strategies

Key-words: Squamous cell carcinoma; ruthenium; metallodrugs; RuMTNZ

BACKGROUND

Non-melanoma skin cancer represents the most frequently diagnosed malignancy worldwide, with approximately 1.32 million new cases projected for 2025, according to GLOBOCAN data monitored by the World Health Organization (WHO). Among its subtypes, squamous cell carcinoma (SCC), a malignant proliferation of the cutaneous epithelium, ranks as the second most common form globally, posing significant clinical challenges due to its aggressive behavior and potential for local invasion (QUE et al., 2025). In particular, head and neck squamous cell carcinoma (HNSCC) frequently presents as locally advanced disease at diagnosis, complicating treatment and adversely affecting prognosis (WANG et al., 2023). Standard management typically involves surgical resection, often in combination with radiotherapy or chemotherapy, yet recurrence rates remain high and survival outcomes are frequently suboptimal.

Cisplatin, first approved in 1978, remains a cornerstone of systemic therapy due to its potent antitumor activity mediated by DNA crosslinking and apoptosis induction (ZHANG et al., 2021). However, upon administration, cisplatin undergoes biotransformation into reactive species that interfere with multiple cellular pathways, contributing to systemic toxicity and limiting its therapeutic window (CURRY; MCCORMICK, 2022). Furthermore, tumor resistance mechanisms—including enhanced DNA repair, increased efflux, and decreased intracellular accumulation—significantly compromise its efficacy (LUGONES; LORE; SALAZAR, 2022). These limitations underscore the urgent need for alternative strategies that combine efficacy, selectivity, and reduced off-target effects.

In this context, metallodrugs based on ruthenium(II) have emerged as promising candidates. Ruthenium complexes offer several pharmacologically advantageous properties over traditional platinum-based agents, including greater chemical stability, lower systemic toxicity, and multiple accessible oxidation states that enable prolonged circulation and selective interaction with tumor cells (D’AMATO et al., 2023). The octahedral geometry of Ru(II) complexes allows diverse modes of DNA interaction and can trigger apoptotic pathways through additional mechanisms, such as lysosomal disruption, making them particularly suited for targeting aggressive and chemoresistant tumors. Notably, the ruthenium(II)–metronidazole (RuMTNZ) complex has been reported to induce cell cycle arrest and promote apoptosis in breast cancer cell lines, demonstrating its potential as a versatile anticancer agent (CÂNDIDO et al., 2022).

Despite these promising attributes, the biological activity of RuMTNZ complexes in models of head and neck SCC remains largely unexplored. The compounds used in the present study were generously provided by Prof. Dr. Carlos Antônio Doriguetto from the Chemistry Institute of the Federal University of Alfenas (UNIFAL-MG). Previous investigations in our laboratory assessed their cytotoxicity in two-dimensional (2D) and three-dimensional (3D) models derived from the FaDu cell line, establishing IC_{50} values and demonstrating that combination with cisplatin did not yield synergistic effects. These findings highlight a critical knowledge gap regarding the independent effects of RuMTNZ complexes on processes central to tumor progression, such as cellular motility, clonal expansion, and proliferative plasticity.

Accordingly, this study aims to investigate the effects of RuMTNZ complexes on fundamental hallmarks of HNSCC biology, including proliferation, migration, and invasion. By elucidating their mechanism of action in this context, we aim to provide critical insights into the therapeutic potential of these complexes as novel metallodrugs capable of overcoming the limitations of conventional platinum-based therapy. Ultimately, this work seeks to establish RuMTNZ as a compelling candidate for the next generation of selective, effective, and low- toxicity treatments for head and neck squamous cell carcinoma.

MATERIAL AND METHODS

Cell Viability

Ruthenium complexes containing metronidazole (RuMTNZ7 and RuMTNZ9) were dissolved in dimethyl sulfoxide (DMSO, Sigma). In previous studies, the IC_{50} was determined using the MTT viability assay in both 2D and 3D cultures. These doses were defined after obtaining dose–response curves, which were subsequently analyzed using the Calcusyn software (Appendix A).

2D Cell Culture and Spheroid Standardization

The FaDu cell line was obtained from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil) and maintained in monolayer culture in 25 cm² flasks containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco), supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and 1% antibiotic solution composed of penicillin (60 mg/mL) and streptomycin (100 mg/mL). Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

For spheroid formation, the protocol adapted from Friedrich et al. (2009) was used. Monolayer cultures were trypsinized upon reaching approximately 90% confluence and seeded at a density of 750 cells per 200 µL into 96-well plates pre-coated with 1.5% agarose (Sigma- Aldrich) to create a non-adherent surface. The plates were then incubated for 72 hours to allow spheroid assembly.

3D Colony Formation Assay

The proliferative capacity of FaDu cells was assessed using a three-dimensional colony formation assay adaptation from Borowicz et al. (2014). Twelve-well plates were first coated with 1 mL of 0.6% agarose and incubated at 4 °C for 40 minutes to allow gelation, followed by 1 hour at 37 °C. A second layer containing 0.3% agarose, DMEM, the respective treatments with RuMTNZ7 or RuMTNZ9 at 8 µM (IC_{50}) and 4 µM (½ IC_{50}), and 2×10^4 FaDu cells per well was then added. After 24 hours, 1 mL of DMEM containing the corresponding treatment for each experimental group was added to maintain optimal culture conditions.

Colonies were allowed to grow for 14 days. Images were acquired using an inverted microscope equipped with an Axio Cam MRc camera (Carl Zeiss) and a 4× objective. Colony diameters were measured using ZEN 2.6 software (Carl Zeiss).

3D Cell Migration Assay

Cell migration 3D was evaluated using tumor spheroids to better mimic the *in vivo* microenvironment, through a protocol adapted from Vinci et al (2013). Spheroids approximately 250 μm in diameter were transferred to 96-well plates pre-coated with 0.1% gelatin (Sigma-Aldrich) to provide mild adhesion while preserving cell motility. Wells were blocked with albumin to establish a suitable chemotactic environment.

Spheroids were treated with RuMTNZ7 or RuMTNZ9 at 8 μM (IC_{50}) and 4 μM . Serum-free DMEM was used to reduce proliferation and nutrient availability, ensuring that migration was evaluated independently of cell growth. Images were acquired every 24 hours over 72 hours using an inverted microscope equipped with a 4× objective and an Axio Cam MRc camera (Carl Zeiss). The migratory area was quantified using ZEN 2.6 software, and the migration rate was calculated as the ratio between the final and initial migrated areas.

Invasion Assay

To evaluate invasive capacity, 1.5×10^5 FaDu cells were seeded into invasion inserts pre-coated with Geltrex™ matrix (Thermo Fisher Scientific), diluted 1:5 in serum-free DMEM. Cells were treated with RuMTNZ7 or RuMTNZ9 at concentrations established in prior assays, while controls received dimethyl sulfoxide (DMSO) only. The lower chamber contained DMEM supplemented with 10% FBS to generate a chemotactic gradient. After 24 hours, invaded cells were fixed with paraformaldehyde and stained with Giemsa. Invasion was quantified using ImageJ software (National Institutes of Health, USA).

Statistical Analysis

All experiments were performed in triplicate to ensure reproducibility and reliability. For the clonogenic assays, each replicate consisted of n = 150 colonies, for migration assays n

= 6 spheroids per condition, and for invasion assays n = 3 inserts per group. Data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Comparisons for clonogenic and invasion assays were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, while migration data were analyzed using two-way ANOVA followed by Bonferroni's post hoc test to account for both treatment and time effects. Results are presented as mean ± standard deviation (SD), and differences were considered statistically significant at $p < 0.05$.

RESULTS

RuMTNZ7 AND RuMTNZ9 SUPPRESS CLONOGENIC GROWTH IN SEMI-SOLID AGAR ASSAYS

To evaluate the effects of RuMTNZ complexes on cell proliferation and their capacity to form colonies, a three-dimensional clonogenic assay was performed and maintained for 14 days, allowing the formation and maturation of tumor spheroids. This assay was selected because it provides a physiologically relevant model that reflects long-term proliferative potential and more closely mimics the in vivo tumor microenvironment compared with traditional 2D assays. At the end of the experiment, colony areas were measured and compared with those of the control group treated with DMSO.

Quantitative analysis revealed that the DMSO-treated control group exhibited an average colony area of 72.84 μm^2, representing the baseline proliferative capacity of FaDu cells under standard culture conditions. Treatment with RuMTNZ7 at 8 μM led to a reduction of approximately 71.78% in colony area compared with the control ($p < 0.0001$), while the lower concentration of 4 μM resulted in a decrease of 76,77% ($p < 0.0001$), indicating a clear dose- dependent antiproliferative effect. Similarly, RuMTNZ9 at 8 μM reduced colony area by 78,28% ($p < 0.0001$), and at 4 μM by 77.11% ($p < 0.0001$), demonstrating comparable efficacy between the two complexes.

These findings suggest that both RuMTNZ7 and RuMTNZ9 interfere with key cellular mechanisms governing proliferation and clonal expansion in FaDu cell line. The observed reduction in colony size has a potential impact on cell survival and the ability of individual cells to initiate and sustain colony formation.

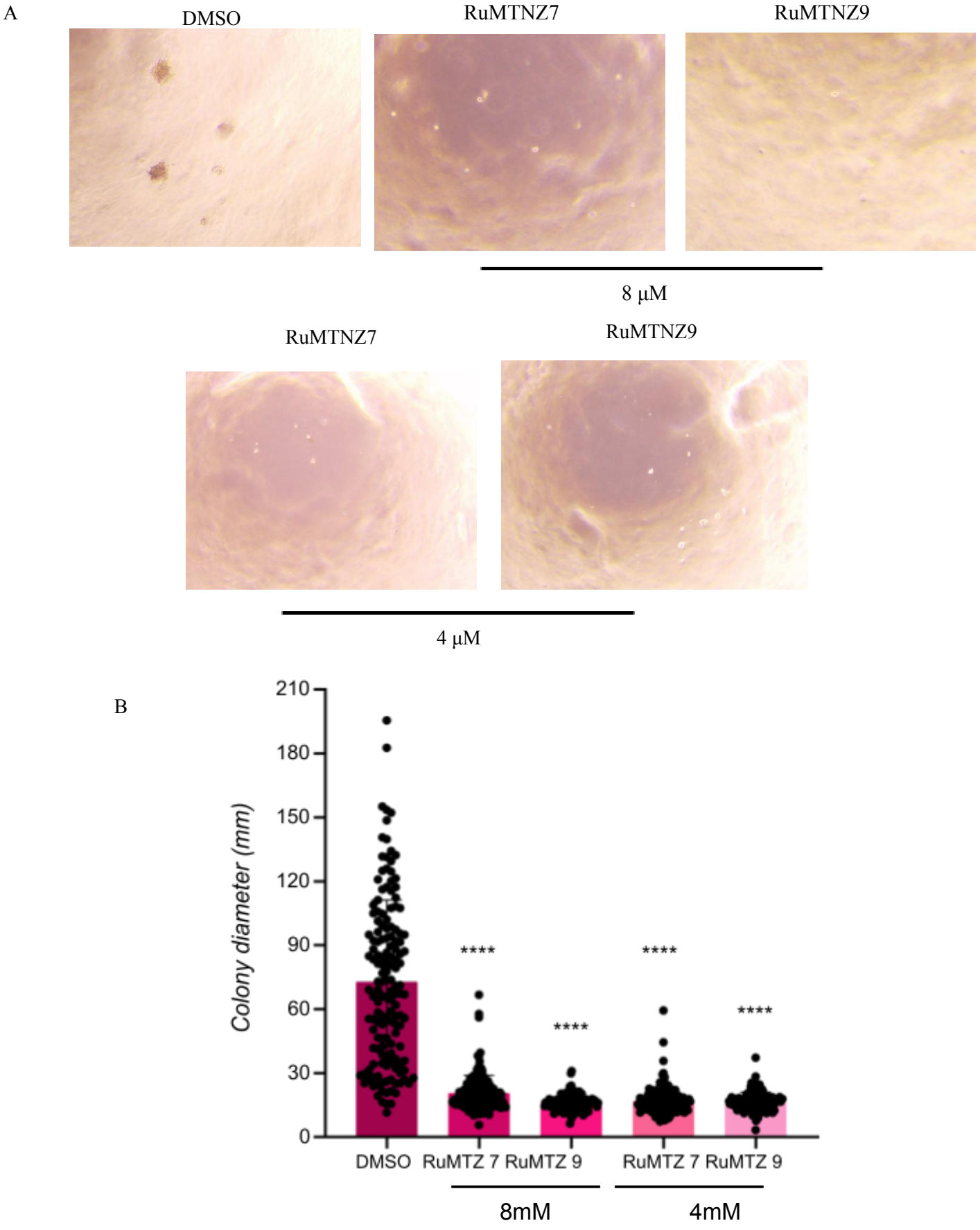


Fig. 1. RuMTNZ complexes reduce colony formation in 3D clonogenic assays. (A) Representative images of colonies formed under DMSO treatment (control) and under exposure to RuMTNZ7 or RuMTNZ9 at 8 µM and 4 µM. Both complexes visibly decreased colony size compared with the control group. (B) Quantification of colony diameter showing that treatments with RuMTNZ7 and RuMTNZ9 at both concentrations significantly reduced colony growth relative to DMSO. Control colonies reached an average diameter of approximately 200 mm, whereas all treated groups displayed markedly smaller diameters ($p < 0.0001$). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

RuMTNZ7 AND RuMTNZ9 EXERT DOSE-DEPENDENT DUAL EFFECTS ON FADU CELL MIGRATION

After confirming the significant reduction in cell proliferation induced by the RuMTNZ complexes, we next evaluated their impact on cell motility using three-dimensional spheroid- based migration assays, which more accurately reproduce the extracellular matrix architecture. The analyses revealed a clear dose-dependent modulation of migratory behavior. Treatment with 8 μM led to a marked decrease in the migrated area compared with the DMSO-treated control group, with mean values of 6,32x (RuMTNZ7); 11.82x (RuMTNZ9) and an overall reduction of 76,92% (RuMTNZ7); 56,66% (RuMTNZ9), which reached statistical significance ($p = 0,0034$; $p = 0.0288$). No meaningful differences were detected between RuMTNZ7 and RuMTNZ9 under this condition, indicating that both complexes exert a comparable inhibitory effect on cell motility.

In contrast, exposure to 4 μM of either RuMTNZ7 or RuMTNZ9 resulted in the opposite outcome, producing a pronounced increase in migration, 21,42% (RuMTNZ7); 28,57% (RuMTNZ9). This unexpected enhancement of migration at subcytotoxic concentrations suggests the potential selection or enrichment of cellular subpopulations with intrinsically higher migratory capacity, highlighting a biphasic and dose-dependent response to RuMTNZ treatment.

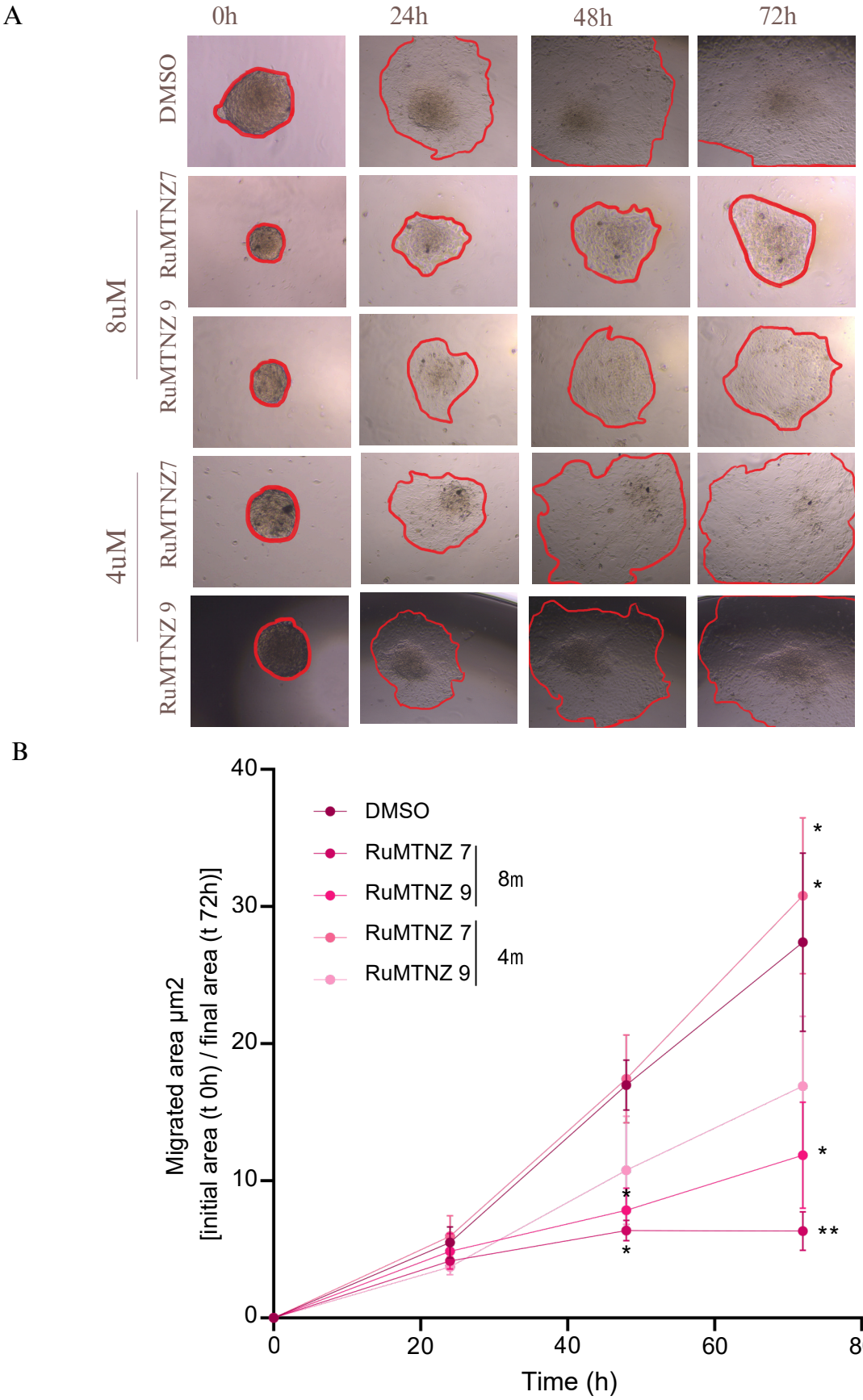


Fig 2. RuMTNZ complexes impair spheroid migration in 3D motility assays. (A) Representative images of FaDu spheroids treated with DMSO (control) or with RuMTNZ7 and RuMTNZ9 at 8 µM (IC_{50}) and 4 µM (½ IC_{50}) over a 72 h period. Red outlines indicate the area migrated beyond the initial spheroid boundary. All RuMTNZ treatments visibly reduced radial expansion compared with the control group. (B) Quantification of migrated area, calculated as the difference between the initial spheroid area (0 h) and the final area (72 h). Both RuMTNZ7 and RuMTNZ9 significantly decreased spheroid migration at all tested concentrations when compared with DMSO, with the most pronounced reductions observed at 72 h. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

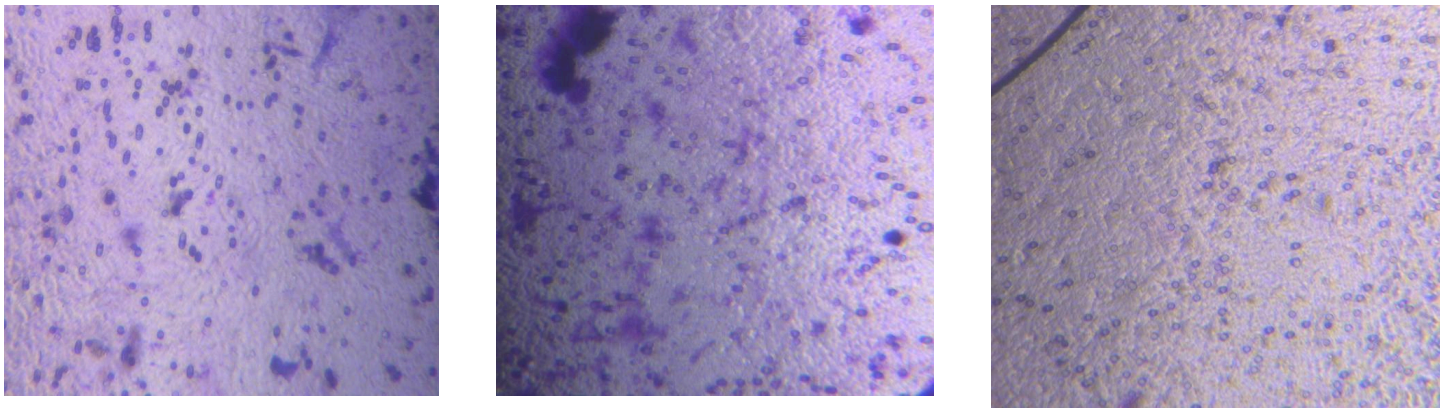
RuMTNZ COMPLEXES DO NOT ALTER THE INVASIVE CAPACITY OF FADU CELLS

After confirming that IC_{50} concentrations of the RuMTNZ complexes were effective in reducing cell migration in three-dimensional assays, we next sought to determine whether these inhibitory effects on motility extended to the cells' ability to invade an extracellular matrix. To this end, we conducted invasion assays using Geltrex™, which provides a biologically relevant mimic of the extracellular matrix (ECM) and imposes a greater structural barrier than traditional two-dimensional substrates.

Only IC_{50} concentrations were evaluated, as subcytotoxic doses did not demonstrate efficacy in modulating migration in previous experiments. Under these conditions, both RuMTNZ7 and RuMTNZ9 exhibited invasion percentages comparable to those of the DMSO- treated control group. Control cells displayed an average invaded area of 31%, whereas RuMTNZ7 and RuMTNZ9 showed values of 33% and 36%, respectively, with no significant differences between the groups.

Together, these findings demonstrate that, although RuMTNZ complexes effectively reduce proliferation and impair migratory capacity at IC_{50} concentrations, they do not significantly alter the ability of FaDu cells to traverse an ECM-like barrier under the conditions tested. This suggests that the pathways governing invasion may be less sensitive—or mechanistically independent—from those modulated by RuMTNZ-driven antiproliferative and antimigratory effects.

A



DMSO RuMTNZ7 RuMTNZ9

B

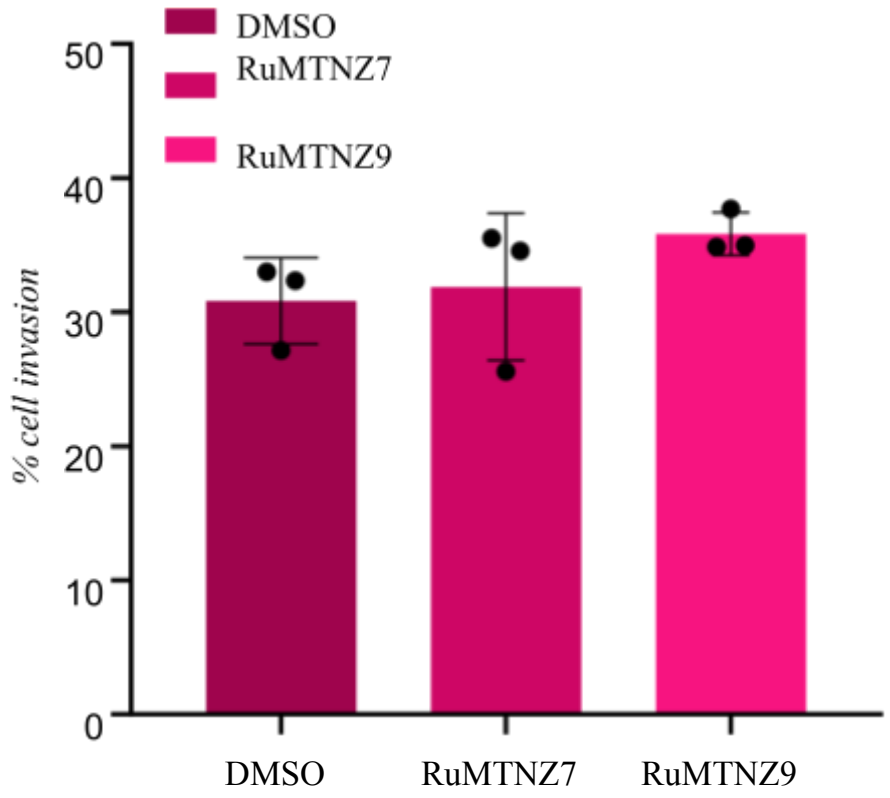


Fig. 3. RuMTNZ complexes do not significantly alter the invasive capacity of FaDu cells. (A) Representative images of the invasion assay performed in Geltrex™ after 24 h of treatment with DMSO (control), RuMTNZ7, or RuMTNZ9. Giemsa staining highlights the cells that successfully traversed the extracellular matrix. (B) Quantification of the percentage of invasive cells. Both RuMTNZ7 and RuMTNZ9 exhibited invasion rates comparable to the control group, indicating no significant modulation of invasive capacity under the tested conditions. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) arises from the squamous epithelium lining the oral cavity and associated structures. Among its subtypes, hypopharyngeal squamous cell carcinoma is particularly aggressive, with a five-year survival rate below 45% (LIN et al., 2022). Its clinical severity is compounded by rapid proliferation and extensive lesion formation, which compromise essential functions of the upper aerodigestive tract, severely affecting patient quality of life and limiting the efficacy of conventional therapies (LEE et al., 2022). Cisplatin remains the gold standard treatment for HNSCC; however, its clinical application is significantly restricted by systemic toxicities, notably nephrotoxicity. This occurs due to preferential accumulation in proximal renal tubular cells, where oxidative stress, inflammation, and apoptosis are triggered, ultimately leading to acute kidney injury (MOTWANI et al., 2022). In addition to systemic toxicity, tumor cells can develop resistance to cisplatin through mechanisms such as enhanced DNA repair, reduced intracellular accumulation, and activation of prosurvival pathways, further limiting its therapeutic efficacy (LUGONES; LOREN; SALAZAR, 2022).

In this context, ruthenium-based complexes have emerged as promising metallodrugs due to their capacity to combine cytotoxic activity with reduced systemic toxicity and improved selectivity toward tumor cells (SIMPSON et al., 2019). Research into ruthenium compounds dates back several decades, driven by the need to develop chemotherapeutic agents that maintain cytotoxic efficacy while minimizing adverse effects. Beyond their antitumor activity, these compounds have been explored for the treatment of parasitic infections such as malaria and leishmaniasis, which further underscores the broad pharmacological relevance and versatility of this class of metallodrugs (BRITTEN & BUTLER, 2022). Among these, the ruthenium–metronidazole complex (RuMTNZ) has previously demonstrated significant capacity to modulate the cell cycle and induce apoptosis in breast cancer models (CÂNDIDO et al., 2022). However, its effects on other tumor types, including hypopharyngeal squamous cell carcinoma represented by FaDu cells, had not been previously investigated, supporting the rationale and scientific relevance of the present study.

After confirming the antiproliferative effect of RuMTNZ complexes, we extended our investigation to their impact on cell motility and invasive potential. Three-dimensional spheroid models were employed to more accurately recapitulate the tumor microenvironment, cellular architecture, and intercellular interactions, which are poorly represented in

conventional two-dimensional cultures (RODRIGO; REIS; PIRRACO et al., 2024). In clonogenic assays, both RuMTNZ7 and RuMTNZ9 significantly reduced colony diameter compared with DMSO-treated controls, confirming robust antiproliferative activity. This finding is consistent with previous evidence demonstrating that Ru(II) complexes effectively suppress colony formation in various cancer models, including colorectal cancer cell lines such as PMC79 and LCR134 (BRÁS et al., 2023) and prostate adenocarcinoma lines such as DU-
145 (DE GRANDIS et al., 2021). These results collectively reinforce the potential of ruthenium-based metallodrugs to impair the long-term survival and self-renewal capacity of tumor cells, highlighting their therapeutic relevance.

Cell motility assays revealed a concentration-dependent effect of the RuMTNZ complexes. At IC_{50} concentrations (8 μM), both RuMTNZ7 and RuMTNZ9 significantly suppressed spheroid migration relative to control. In contrast, subcytotoxic concentrations (4 μM) failed to sustain this inhibitory effect, with cells exhibiting migratory behavior comparable to untreated controls and expansion of the migrated area by up to 36-fold. This phenomenon suggests that at sublethal doses, the complexes may not uniformly affect heterogeneous tumor populations, allowing survival and expansion of subpopulations with higher intrinsic migratory potential. Similar observations have been reported with cisplatin, where low-dose treatment induces an epithelial–mesenchymal transition (EMT)-like phenotype and enhances migration and invasion in lung adenocarcinoma cells (Zhang et al., 2022). These results emphasize the importance of evaluating concentration-dependent effects when assessing the therapeutic potential of metallodrugs, and underscores the scientific need to employ a 3D cell-culture model to achieve a more faithful and physiologically relevant characterization of the therapeutic response, more closely recapitulating the in vivo tumor milieu.

To further delineate the effects of cytotoxic concentrations, the boyden chamber invasion assay was performed using Geltrex™ as an extracellular matrix mimic. Only IC_{50} concentrations were applied, as subcytotoxic doses had no observable impact on migration. Interestingly, neither RuMTNZ7 nor RuMTNZ9 significantly altered the invasive capacity of FaDu cells, with invaded areas comparable to DMSO-treated controls (control: 31%; RuMTNZ7: 33%; RuMTNZ9: 36%), with no significant differences between the groups. These findings suggest that while RuMTNZ complexes effectively reduce migration at cytotoxic doses, this effect does not extend to invasion through an extracellular matrix barrier. In light of these observations and the migration assay results, we hypothesize that the RuMTNZ complexes may alter cytoskeletal organization, membrane dynamics, or other motility-related pathways

without substantially affecting the extracellular matrix degradation or remodeling mechanisms required for invasion. As reported by Schaeffer et al. (2014) in prostate adenocarcinoma, simultaneous induction of factors that promote both migration and invasion resulted in a decoupled response, demonstrating that these processes can be regulated through independent mechanisms.

Taken together, our findings highlight the therapeutic potential of RuMTNZ7 and RuMTNZ9 for head and neck squamous cell carcinoma. The use of three-dimensional models enabled a detailed evaluation of clonogenic capacity and migratory behavior, while invasion assays offered complementary quantitative insights. Although the complexes did not modulate invasion, their pronounced effects on proliferation and migration at cytotoxic concentrations indicate that they can selectively target specific aspects of tumor aggressiveness. The distinct responses observed at subcytotoxic doses further underscore the complexity of tumor plasticity and intratumoral heterogeneity, suggesting that therapies relying solely on cytotoxic mechanisms may be insufficient to fully limit tumor progression. Overall, these results reinforce the need for integrated therapeutic strategies that consider the multifaceted biology of squamous cell carcinoma and support future investigations combining cytotoxic and anti- motility approaches to enhance clinical efficacy

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5. CONCLUSÃO

Em conclusão, este estudo avaliou o potencial terapêutico dos complexos de rutênio(II) associados ao metronidazol (RuMTNZ7 e RuMTNZ9) frente ao carcinoma espinocelular hipofaríngeo, uma neoplasia altamente agressiva cujo tratamento é limitado pela toxicidade associada à cisplatina.

Por meio da combinação de ensaios tridimensionais, mais representativos da arquitetura tumoral, com ensaios bidimensionais voltados à análise funcional, foi possível caracterizar de forma abrangente os efeitos biológicos desses compostos. Nos modelos 3D, ambos os complexos reduziram significativamente a formação e o diâmetro das colônias, indicando impacto direto sobre a proliferação das células tumorais. De maneira complementar, os ensaios de migração demonstraram que concentrações citotóxicas de RuMTNZ7 e RuMTNZ9 suprimem de forma expressiva o deslocamento celular, sugerindo potencial efeito antimetastático.

Por outro lado, os complexos não alteraram a capacidade invasiva das células, e concentrações subcitotóxicas foram insuficientes para modular a motilidade celular. Esse padrão reflete o observado em outros modelos tumorais, nos quais proliferação, migração e invasão podem responder de maneira dissociada aos estímulos terapêuticos. Tais achados ressaltam a complexidade da plasticidade fenotípica tumoral e evidenciam que atuar em um único eixo biológico raramente é suficiente para interferir em todos os aspectos da progressão tumoral.

Em suma, o presente estudo contribui para o avanço do conhecimento sobre o uso de complexos de rutênio no carcinoma espinocelular de cabeça e pescoço, demonstrando sua eficácia em reduzir a proliferação e a migração celular sob condições citotóxicas, reforçando seu potencial como candidatos promissores na classe dos metalofármacos. Estes resultados estabelecem uma base sólida para investigações futuras, incluindo abordagens combinatórias e estudos mecanísticos mais aprofundados, com vistas ao desenvolvimento de terapias mais eficazes e menos tóxicas para pacientes acometidos por essa neoplasia.

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ANEXO 01- ANÁLISE DAS VIABILIDADES

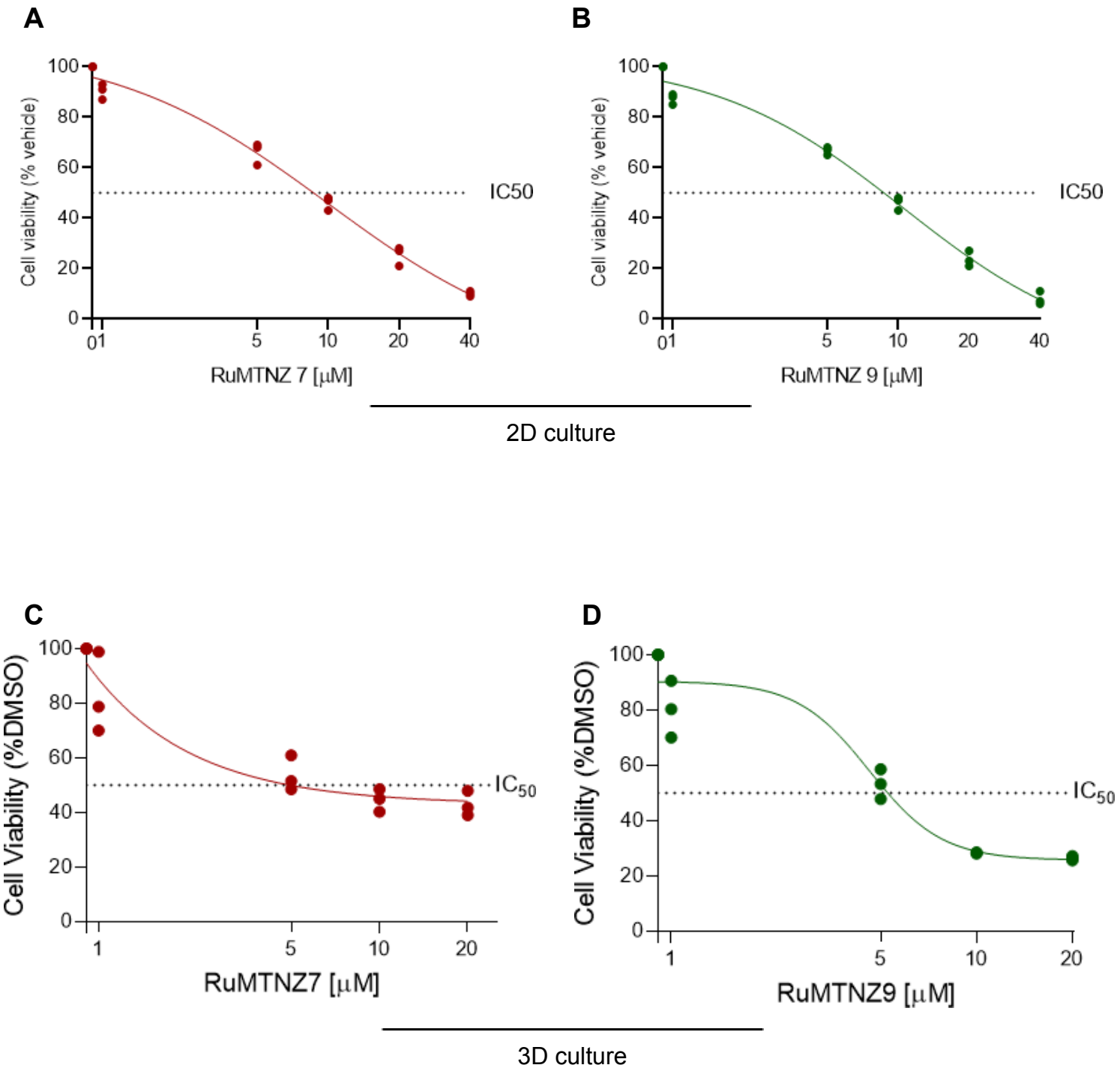

Fig. 1. Evaluation of FaDu cell viability . Cells were exposed to RuMTNZ-7 **(A)**, RuMTNZ-9 **(B)**, in 2d culture, and RuMTNZ-7 (C), RuMTNZ-9 (D), in 3D culture