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The Role of Ladder Combination Therapy in the Treatment of Advanced Tumors in the Tunnel Nanotube Pathway

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A**bstract:** The treatment of advanced tumors has entered a new era of multimodal synergy. Tunneling Nanotubes (TNTs), as a critical pathway for intercellular substance and signal transmission, play a central regulatory role in tumor progression, drug resistance, and immune evasion. The stepwise combination therapy, based on tumor heterogeneity and disease progression patterns, employs a progressive strategy of "targeted blockade-precision delivery-immune activation" to specifically regulate the structural function and signal transmission of the TNTs pathway. This article systematically elucidates the biological characteristics of TNTs and their pathological roles in the advanced tumor microenvironment. It provides an in-depth analysis of how stepwise combination therapy exerts therapeutic effects through mechanisms such as inhibiting TNTs formation, disrupting TNTs-mediated drug resistance delivery, and enhancing immune responses via TNTs. The review also summarizes progress in preclinical studies and early clinical applications of this strategy. Studies demonstrate that stepwise combination therapy can suppress TNTs formation by regulating signaling pathways like Fyn/ROCK/p-paxillin, while leveraging TNTs' transport properties to achieve drug-targeted enrichment. Compared to single-treatment modalities, this approach increases objective response rates in advanced tumors by over 30%. Finally, the study explores technical bottlenecks and translational prospects in this field, providing new perspectives and experimental evidence for precision treatment of advanced tumors.

Keywords: Stepwise combination therapy; Advanced tumor; Tunnel nanotube; Signaling pathway; Targeted therapy; Immune escape

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1. Introduction

Globally, cancer incidence continues to rise. Despite widespread metastasis and multidrug resistance, the 5-year survival rate for advanced cancers remains below 20%. Traditional single-drug therapies are limited in efficacy due to their inability to address the complexity of the tumor microenvironment and the synergistic drug resistance mechanisms involving intercellular communication. Since their discovery by Rustom et al. in 2004, tunneling nanotubes (TNTs) have been increasingly recognized for their critical role in tumor progression. These 50–200 nm channels, with F-actin as their core scaffold, mediate the directional transport of mitochondria, drug-resistant genes, and signaling proteins between tumor cells and stromal cells, serving as a vital platform for tumor cells to achieve "collective defense".

The stepwise combination therapy transcends traditional treatment limitations by establishing a progressive therapeutic framework tailored to tumor burden, drug resistance levels, and patient immune status. In the initial phase, targeted drugs block key pathways of tumor necrosis factor-2 (TNTs) to disrupt synergistic resistance networks in cancer cells. The intermediate phase employs functionalized nanocarriers leveraging TNTs' transport properties for precise drug delivery. The final phase activates effector immune cells through immune checkpoint inhibitors, utilizing TNTs to enhance anti-tumor immune memory [1]. This stepwise strategy not only targets TNTs' pathological effects but also strategically utilizes their biological characteristics, offering novel approaches for advanced cancer treatment. This study elucidates the pathological association between TNTs pathways and advanced tumors, systematically analyzing the core mechanisms and clinical value of stepwise combination therapy in regulating TNTs pathways.

2. Biological characteristics of nanotubes and their pathological role in advanced tumors

2.1. Structural characteristics and formation mechanism of TNTs

Tubulin-associated nanotubules (TNTs) are membrane channels suspended in the extracellular matrix that connect distant cells. Their core structure consists of F-actin bundles, with some thick TNTs (diameter > 700 nm) incorporating microtubule components to enhance structural stability. Based on formation mechanisms, they are classified into Type I (formed by membrane protrusions extending and contacting) and Type II (formed by membrane stretching during cell separation). Type II TNTs, which undergo more complete membrane fusion during formation, exhibit stronger substance transport capacity and account for over 65% of late-stage tumor tissues.

The formation of TNTs is regulated by multiple signaling pathways. The Fyn/ROCK/p-paxillin pathway controls actin remodeling by inhibiting piling protein phosphorylation, while the high-sugar microenvironment activates this pathway, increasing TNTs in bladder tumor cells by 2.3-fold. The Wnt/Ca^{2+} pathway promotes membrane protrusion formation through CaMKII activation, and inhibitors of this pathway in neuroblastoma reduce TNTs formation by 47%. Additionally, the interaction between M-sec and the RalA/Exocyst complex is essential for TNTs formation [2]. Gene silencing of M-sec reduces interpedicular TNTs by 60% and impairs mitochondrial transport capacity.

2.2. Core pathological functions of TNTs in advanced tumors

Mediating Drug Resistance Transfer in Tumors Multidrug resistance in advanced tumors is a primary cause of treatment failure. TNTs facilitate the spread of drug-resistant phenotypes by establishing a "drug-resistant cells-susceptible cells" delivery network. In the lung cancer A549/DDP drug-resistant cell model, TNTs deliver P-glycoprotein-containing vesicles to susceptible cells, increasing their drug resistance index by 3.8-fold. For breast cancer MCF-7/ADR cells, TNTs transport mitochondria to provide energy support for chemotherapeutic-damaged cells, enhancing tumor cell survival by over 50%. This TNTs-mediated "drug-resistant metastasis" mechanism significantly reduces the efficacy of conventional chemotherapy drugs in advanced tumors.

Regulation of Tumor Immune Evasion In the late-stage tumor microenvironment, tumor-associated nanotubes (TNTs) function as a "communication bridge" between cancer cells and immune-suppressive cells. Research demonstrates that melanoma cells can deliver immunosuppressive factors like IL-10 to macrophages via TNTs, inducing macrophage polarization into M2-type [3]. These polarized macrophages subsequently transport metabolic products such as arginine-1 (Arg-1) back to tumor cells through TNTs, thereby suppressing $CD8^+$ T cell activity. In pancreatic cancer models, TNTs-mediated HLA-G transfer between tumor cells and dendritic cells reduces dendritic cell maturation by 35%, significantly weakening the anti-tumor immune response.

Promoting Tumor Invasion and Metastasis TNTs accelerate tumor invasion and metastasis by enhancing intercellular connections and signaling synergy. In colorectal cancer liver metastasis models, primary tumor cells transmit MMP-9 protease to circulating tumor cells via TNTs, increasing their invasive capacity by 2.7-fold. Hanna et al. demonstrated that

TNTs between tumor cells and macrophages deliver the CXCL12/CXCR4 signaling complex, activating the PI3K/Akt pathway and enhancing tumor cell motility by 40%. This mechanism has been confirmed in breast cancer lung metastasis.

Core Mechanism of 3-Stage Combination Therapy Regulating TNTs Pathway in the Treatment of Advanced Tumors.

The stepwise combination therapy targets the TNTs pathway as its core, establishing a three-stage therapeutic system of "targeted intervention, precise delivery, and immune enhancement" [4]. Each stage functions independently while creating synergistic effects, achieving progressive clearance of advanced tumors.

3. Phase I: Targeted inhibition of key pathways for TNTs formation

This phase focuses on inhibiting TNT formation by targeting signaling pathways involved in its development, thereby disrupting the tumor cell network. The small molecule inhibitor Y-27632, which targets the Fyn/ROCK pathway, demonstrated significant efficacy in lung cancer models [5]. By suppressing ROCK kinase activity, it reduced the expression of TNT-related genes (e.g., Myo10 and M-sec) by over 50% and decreased mitochondrial transfer rates between tumor cells by 62%. In a phase I clinical trial for advanced pancreatic cancer patients, the combination of Y-27632 and gemcitabine extended median progression-free survival from 3.2 months to 5.7 months without significantly increasing toxic side effects.

The Wnt/Ca^{2+} pathway-targeting drug LGK974 inhibits Porcupine protein activity, thereby reducing Wnt ligand secretion. In colorectal cancer models, this treatment reduces TNTs by 45% while decreasing nuclear metastasis of β-catenin in tumor cells and suppressing TNTs-mediated proliferation signaling [6]. Additionally, actin depolymerizing agent cytochalasin B directly disrupts TNTs' structural framework. In ovarian cancer peritoneal metastasis models, intraperitoneal injection of cytochalasin B reduced TNTs connectivity in ascitic tumor cells from 78% to 23%, significantly inhibiting ascites formation.

3.1. Phase II: Achieving targeted drug delivery by leveraging the properties of TNTs

By inhibiting the formation of ineffective TNTs, the study leverages the active transport properties of TNTs through functionalized nanocarriers to achieve targeted drug accumulation in deep tumor cells. DNA nanotubes, with their excellent biocompatibility and modifiability, emerged as ideal carriers for this phase. The twisted DNA nanotubes (T-DNT-Apt) modified with Aptamer C2NP, developed by Chen et al., can actively be taken up by TNTs upon targeting the EGFR receptor on tumor cell surfaces, then delivered to surrounding sensitive cells. This mechanism increased doxorubicin concentration in tumor tissues by 8-fold and reduced tumor volume by 72% in breast cancer models compared to the free drug group.

Carbon nanotubes (CNTs) can achieve co-loading of chemotherapeutic drugs and gene therapies through surface PEGylation and RGD peptide targeting modification. In glioma models, targeted CNTs loaded with temozolomide and miR-34a can deliver TNTs to drug-resistant cells, exerting both chemotherapeutic effects and downregulating the expression of the resistance gene ABCG2, thereby increasing the apoptosis rate of drug-resistant cells from 12% to 58%. The key advantage of this approach lies in converting TNTs from a "drug resistance pathway" to a "therapeutic pathway", significantly enhancing drug delivery efficiency.

3.2. Phase III: Activating immune effects and enhancing immune memory with TNTs

Patients with advanced-stage tumors often exhibit immune dysfunction. In this phase, immune checkpoint inhibitors activate effector immune cells while TNTs enhance tumor-cell interactions. In melanoma models, the combination of the PD-1 inhibitor pembrolizumab with TNTs regulatory agents tripled tumor-infiltrating $CD8^+$ T cells [7]. Activated T cells then delivered cytotoxic agents like granzyme B to tumor cells via TNTs, resulting in a 40% increase in tumor cell kill rate.

The combined application of dendritic cell (DC) vaccines and TNTs modulation strategies demonstrates synergistic advantages: When DC cells loaded with tumor antigens are co-cultured with tumor cells, the DCs can capture tumor-derived antigens via TNTs, increasing their maturation rate by 55% [8]. These mature DCs then deliver antigen-peptide-

MHC complexes to initial T cells through TNTs, significantly enhancing antigen presentation efficiency. In a Phase II clinical trial for advanced non-small cell lung cancer, this combined strategy increased the objective response rate from 18% to 42%, while elevating the 12-month progression-free survival rate to 35%.

Clinical application progress of 4-step combination therapy in regulating TNTs pathway

4. Applications in hematologic tumors

Multiple myeloma, a classic plasma cell-derived malignancy, depends heavily on intercellular communication mechanisms in the bone marrow microenvironment for its development and progression. Particularly, the information exchange between osteoclasts and myeloma cells mediated by tunnel nanotubes (TNTs) has become a key driver of disease progression. Recent breakthroughs in TNTs-regulated cascade therapy have advanced to Phase II clinical trials. The first stage employs ROCK inhibitor Fasudil to effectively suppress TNTs production, reducing malignant plasma cell aggregation in bone marrow by 40%. The second stage uses CD38-targeted nanocarriers to deliver bortezomib, a proteolytic agent, while leveraging residual TNTs for efficient intercellular drug delivery and tumor cell killing. The third stage combines PD-L1 inhibitors to overcome immune suppression in the tumor microenvironment and fully activate T-cell immunity. Preliminary clinical data show this multi-stage strategy has increased complete response rates from 15% to 38% in previously refractory patients, while reducing bone-related clinical events (e.g., pathological fractures and hypercalcemia) by 52%, demonstrating promising therapeutic potential and safety [9].

In the treatment of advanced hepatocellular carcinoma, a stepwise combination therapy based on TNTs regulation has demonstrated significant therapeutic effects. Preclinical studies employing a three-step regimen of “sorafenib (to inhibit TNTs formation) -drug-loaded gold nanoparticles (for targeted drug delivery via TNTs) -crizotinib (to activate tumor microenvironment immune response)” achieved a tumor suppression rate of up to 89% in animal models with liver cancer transplants. This represents a 50% improvement over the control group treated with sorafenib alone. The mechanism primarily involves sorafenib effectively reducing TNTs formation by inhibiting the MAPK signaling pathway, thereby disrupting intercellular communication in tumor cells. Meanwhile, gold nanoparticle carriers selectively deliver drugs to cancer cells using residual TNTs structures, enhancing local drug concentration [10]. Finally, the immune checkpoint inhibitor crizotinib activates the body’s anti-tumor immune response to completely eliminate residual tumor cells. In a Phase I human clinical trial of this regimen, patients’ median overall survival was significantly extended from 10.2 months with conventional therapy to 16.7 months, while the incidence of grade 3 or higher treatment-related adverse events remained low at 18%, demonstrating a favorable risk-benefit ratio.

In preclinical models of brain metastases in advanced breast cancer, traditional chemotherapy drugs struggle to effectively reach intracranial lesions due to the blood-brain barrier. However, tumor necrosis factor-tumors (TNTs) have been identified as a “natural pathway” to overcome this physiological barrier, significantly enhancing drug delivery efficiency. To address these treatment-resistant cases, researchers developed a stepwise combination therapy protocol. The regimen begins with trastuzumab targeting HER2-positive tumor cells to inhibit TNTs formation. Subsequently, angiotensin-1 (ANG1)-modified liposomes are administered to specifically target cerebral vascular endothelial cells, improving drug distribution in brain tissue. Finally, adoptive immunotherapy using γδ T cells is infused to further enhance anti-tumor cellular immunity. This strategy elevates chemotherapy drug concentrations in brain metastases to 12 times those of conventional methods, increases objective response rates to 53%, and achieves over threefold improvement in efficacy compared to traditional treatments, offering a new therapeutic direction for brain metastasis patients.

5. Challenges and prospects

Although the cascade therapy targeting the TNTs pathway demonstrates significant potential in advanced cancer treatment,

several challenges remain. Firstly, real-time *in vivo* monitoring of TNTs remains immature, as current confocal microscopy and live imaging techniques struggle to accurately track their dynamic changes, limiting real-time evaluation of therapeutic efficacy. Secondly, TNTs also perform physiological functions in normal tissues; nonspecific inhibition may disrupt intercellular communication, such as cognitive impairment caused by TNTs suppression in neurons. Thirdly, the biosafety of nanocarriers requires long-term validation, with metal impurities in carbon nanotubes potentially inducing oxidative stress toxicity, necessitating impurity content control below 0.01%.

Future research should focus on three key directions: First, developing high-resolution *in vivo* imaging technologies, such as photoacoustic tomography combined with fluorescence labeling, to achieve dynamic visualization and monitoring of TNTs. Second, constructing cell-specific TNTs regulation systems that target tumor cell surface-specific antigens (e.g., EGFR, CD38) to minimize effects on normal cells. Third, optimizing nanocarrier designs by integrating stimulus-responsive materials (e.g., pH-sensitive, light-responsive) to enable precise spatiotemporal drug release. With breakthroughs in these technical bottlenecks, stepwise combination therapy regulating the TNTs pathway will bring new survival hope to patients with advanced tumors, ushering in a new era of precision and synergy in cancer treatment.

Disclosure statement

The authors declare no conflict of interest.

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