Validated CT26 Allograft Model:

Subcutaneous, Orthotopic, And Metastatic Allograft Tumor Model

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Advancing Colorectal Cancer Research with the CT26 Allograft Model

Colorectal cancer remains a leading cause of cancer-related morbidity and mortality worldwide, with limited therapeutic success in advanced stages despite progress in molecular diagnostics and treatment strategies. The inherent complexity of tumor heterogeneity, immune evasion, and resistance to therapy has emphasized the need for preclinical models that closely reflect the biological and immunological features of the disease. Allograft models, particularly syngeneic allografts such as the CT26 colon carcinoma model in BALB/c mice, provide a robust platform for investigating tumor-immune dynamics within an immunocompetent host. These models offer advantages over human xenografts in immunodeficient mice by enabling the study of immune checkpoint blockade, cytokine signaling, and tumor-infiltrating lymphocyte function. However, challenges persist, including limited genomic diversity and reduced fidelity to human tumor microenvironments. Addressing these limitations, the current research employs the CT26 allograft model to examine immunological correlates of therapeutic response, with the objective of advancing the predictive capacity of syngeneic systems and contributing to the development of more effective and personalized colorectal cancer treatments.

CT26 Cell Line

The CT26 murine colon carcinoma cell line, originally derived from a chemically induced tumor in BALB/c mice, is extensively utilized in preclinical cancer research as a syngeneic model of microsatellite stable colorectal cancer. It is characterized by a KrasG12D mutation and exhibits high immunogenicity, with a tumor microenvironment rich in cytotoxic T lymphocytes, macrophages, and myeloid-derived suppressor cells. CT26 has become a cornerstone in evaluating the efficacy of immunotherapies, including checkpoint inhibitors, adoptive T cell transfer, and mRNA-based vaccines. Studies have demonstrated partial sensitivity of CT26 tumors to PD-1 blockade, with enhanced responses observed when used in combination with agents targeting innate immunity or costimulatory pathways. The model has also supported the development of nanoparticle-mediated drug delivery systems and oncolytic virotherapies. However, its artificial origin and murine-specific oncogenic profile limit its translational applicability, particularly regarding mechanisms of immune evasion and tumor-stroma interactions observed in human colorectal cancers. Furthermore, discrepancies in immunotherapy responsiveness across experimental conditions underscore the need for deeper characterization of the tumor immune landscape within this model.

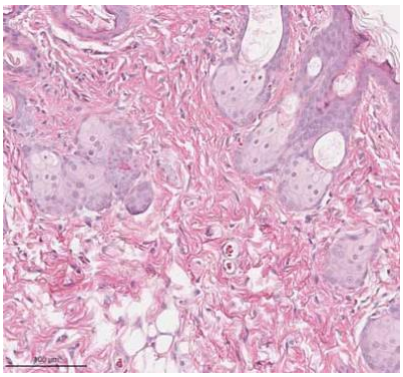

Figure 1. Tumor Histology. H&E stained section of a subcutaneously-implanted CT26 tumor (Altogen Labs).

Altogen Labs Validated CT26 Allograft Model

CT26 cells are maintained under exponential growth and prepared for injection through trypsinization, with viability confirmed by trypan blue exclusion requiring a minimum of 98 percent viable cells. Cell suspensions are adjusted to the proper density and injected subcutaneously into the hind flank of 10-week-old NCr-nu/nu mice at a concentration of one million cells in 100 microliters of a Matrigel and CT26 suspension. Injection sites are monitored and palpated multiple times weekly until tumors are established, after which tumor size is tracked using digital calipers until reaching 50 to 150 mm³. Once tumors reach the target size, animals are randomized into treatment groups, and the compound of interest is administered following the designated dosing schedule.

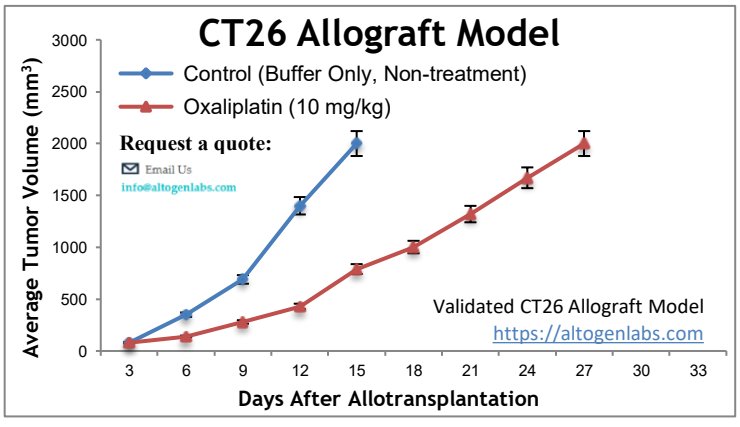

Figure 2. Tumor growth curve of CT26 colon cancer allografts in immunocompromised mice. Data shown as mean ± SEM. Study conducted by Altogen Labs.

Tumor measurements and animal weights are recorded up to three times per week. Animals are euthanized when tumor burden approaches 2,000 mm³ or meets study-defined endpoints. At termination, tumors are excised, weighed, and photographed, followed by gross necropsy and tissue collection for downstream analysis. Tumor and tissue samples can be preserved in RNA Later, snap frozen, or processed for histological evaluation. Altogen Labs supports preclinical oncology studies through the validated CT26 syngeneic colon cancer allograft model, designed for evaluating antitumor efficacy, immunotherapy combinations, and gene modulation strategies. With a comprehensive suite of services that includes over 90 CDX models and more than 30 patient-derived xenograft (PDX) models, Altogen enables translational research aimed at characterizing the molecular and cellular mechanisms driving tumor progression and therapeutic resistance. The CT26 model is leveraged to assess immune checkpoint inhibitors, viral vector-based therapies, and targeted agents in the context of a fully immunocompetent murine host. Altogen also offers specialized services in RNA interference, stable cell line generation, protein expression profiling, and *in vivo* toxicology, providing researchers with a robust platform for mechanistic studies and drug development initiatives.

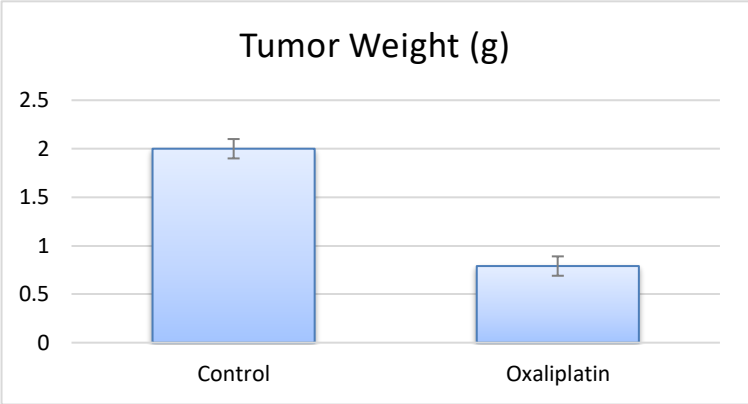

Figure 3. Tumor weight of CT26 allografts on Day 15 in control (buffer-treated) and oxaliplatin-treated mice (10 mg/kg/day), demonstrating significant tumor growth inhibition in the treatment group (Altogen Labs).

Subcutaneous Transplantation of CT26 for Immune Response Assessment

Subcutaneous allograft transplantation using the CT26 murine colon carcinoma model represents a widely utilized and well-characterized approach in preclinical cancer research. This model offers distinct advantages, including consistent tumor engraftment, ease of tumor measurement, and compatibility with immunocompetent BALB/c mice, allowing researchers to evaluate immune-mediated responses within a physiologically relevant context. Unlike allografts established in immunodeficient hosts, the CT26 model preserves the functional integrity of the immune system, enabling the assessment of immunotherapies such as checkpoint inhibitors, cytokine-based treatments, and oncolytic agents. Studies such as those by Lechner et al. have highlighted the value of this model in characterizing tumor-infiltrating lymphocytes and identifying immune biomarkers predictive of therapeutic response.

While orthotopic and metastatic models offer site-specific insights, subcutaneous transplantation remains essential for standardized efficacy studies and mechanistic investigations. The accessibility of subcutaneous tumors permits longitudinal monitoring and repeated sampling, facilitating downstream analyses including flow cytometry, histology, and gene expression profiling. Research by Zhao et al. has shown that despite anatomical differences, subcutaneous CT26 tumors can support robust immune activation, particularly when combined with immunomodulatory agents. Although limitations exist regarding the lack of organ-specific microenvironments, the CT26 subcutaneous model remains a critical platform for early-stage therapeutic evaluation and continues to inform the rational development of immuno-oncology strategies.

Orthotopic CT26 Models for Colorectal Cancer Research

Orthotopic allograft transplantation provides a biologically relevant platform for modeling tumor growth, immune interactions, and therapeutic response within the anatomical site of origin. In colorectal cancer research, the CT26 cell line can be orthotopically implanted into the cecal wall of syngeneic BALB/c mice, creating a tumor microenvironment that closely mimics the structural and immunological features of primary colon tumors. Unlike subcutaneous models, orthotopic transplantation supports the development of tissue-specific vasculature and facilitates interactions with regional immune cells, fibroblasts, and extracellular matrix components. This approach is particularly valuable for evaluating tumor progression, local invasion, and the mechanisms driving site-specific metastasis, which are often not recapitulated in ectopic models.

The CT26 orthotopic model offers a unique opportunity to investigate immune-based therapies within a physiologically relevant setting. Its compatibility with immunocompetent hosts enables rigorous assessment of tumor-immune dynamics, including T cell infiltration, antigen presentation, and cytokine signaling within the gastrointestinal microenvironment. This model is well-suited for studying the influence of the tumor niche on therapeutic outcomes and for identifying factors that contribute to immune evasion and treatment resistance.

Metastatic Progression and Immune Response in CT26 Models

Metastatic allograft transplantation is critical in preclinical oncology, providing a platform to investigate the biological processes underpinning cancer dissemination and to evaluate therapies aimed at preventing or treating metastatic disease. The CT26 murine colon carcinoma model, widely used in colorectal cancer research, is particularly well-suited for generating metastatic allograft systems due to its aggressive growth kinetics and immunogenic profile. When introduced into syngeneic BALB/c mice, CT26 cells can give rise to secondary tumor sites through both spontaneous and experimental routes of metastasis, enabling researchers to study organ-specific colonization patterns and immune responses in an immunocompetent setting.

Metastatic CT26 models are typically established through either orthotopic implantation followed by natural dissemination or intravenous injection to target specific metastatic sites such as the lungs. These approaches allow for reproducible assessments of metastatic potential and therapeutic response. Moreover, the use of luciferase-expressing CT26 variants facilitates real-time, non-invasive monitoring of tumor burden and metastatic progression. This model system supports detailed immunological and molecular characterization of metastasis, including the analysis of immune suppressive pathways, cytokine signaling, and stromal interactions. By capturing key features of the metastatic cascade within a syngeneic environment, CT26 metastatic models offer a valuable preclinical framework for evaluating novel interventions and improving therapeutic strategies for advanced-stage colorectal cancer.

Case Study: ATR Inhibition and Immune Activation in CT26 MMR-Deficient Tumors

A study by Wang et al., published by *Genes & Development* journal, investigated the therapeutic potential of ATR inhibition in mismatch repair-deficient (MMR-d) colorectal cancer using the CT26 syngeneic mouse model. Key findings demonstrate that CT26 cells lacking MLH1 exhibit heightened sensitivity to ATR inhibitors, such as AZD6738, even in the absence of pronounced microsatellite instability. *In vitro* assays showed reduced viability and increased DNA damage in Mlh1-deficient CT26 cells, particularly during replication, with the damage requiring MUS81 endonuclease activity. These results suggest a synthetic lethal interaction between MMR deficiency and replication stress. Immunofluorescence analysis revealed that ATRi-induced DNA damage colocalized with replication forks and MMR proteins such as PCNA and MSH2. A CRISPR screen identified an increased dependency on ATR and its regulatory partners in Mlh1-deficient cells, reinforcing the role of ATR as a key vulnerability in the absence of functional MMR machinery.

In vivo experiments using immunocompetent BALB/c mice bearing CT26 tumors confirmed the selective efficacy of ATR inhibition in Mlh1-deficient xenografts. While ATRi partially reduced tumor burden under CD8-depleted conditions, optimal antitumor effects required intact T cell immunity, indicating that immune activation contributes to therapeutic response. Mechanistically, ATRi treatment led to cytosolic DNA accumulation, activation of the cGAS-STING pathway, and upregulation of type I interferon signaling in Mlh1-deficient CT26 tumors. Combination therapy with ATRi and anti-PD-1 blockade resulted in synergistic tumor suppression and prolonged survival, an effect not observed in MLH1-proficient tumors. The study's strength lies in its integration of molecular, genetic, and immunological analyses across *in vitro* and *in vivo* platforms. While the CT26 model offers high translational relevance, further work is needed to explore resistance mechanisms, validate findings in orthotopic or metastatic settings, and assess the strategy in MSI-low or immunologically cold tumor contexts. These results highlight the value of CT26 in modeling immune-stimulatory synthetic lethality and provide a rationale for combining ATR inhibition with immunotherapy in MMR-deficient colorectal cancers.

Additional Case Study: Targeting Colorectal Cancer Growth via SIRT2 Upregulation in CT26 Models

Another study by Li et al., published by *Frontiers in Pharmacology* journal, presents compelling evidence that decylubiquinone (DUb), a coenzyme Q10 analog, inhibits colorectal cancer growth through the upregulation of SIRT2. Using the CT26 allograft model, a syngeneic and immunocompetent murine system, the researchers demonstrated that daily administration of DUb significantly reduced tumor volume and weight without inducing systemic toxicity. Immunohistochemical analysis revealed decreased Ki67 expression, indicating reduced tumor proliferation. These results were consistent with findings in patient-derived allograft models and *in vitro* assays using HCT116 and LoVo colorectal cancer cell lines. DUb suppressed proliferation, colony formation, migration, and invasion, but did not increase apoptosis, suggesting a cytostatic rather than cytotoxic mechanism.

Central to the findings was the identification of SIRT2 as a key mediator. DUb treatment markedly increased both mRNA and protein expression of SIRT2 in CT26 allografts. Functional silencing of SIRT2 abrogated DUb's inhibitory effects on proliferation and migration, confirming its mechanistic relevance. While the use of CT26 provided a robust and immunologically relevant platform, limitations include a short treatment window and the absence of long-term resistance

profiling. Nevertheless, the study underscores the therapeutic potential of DUb and validates the CT26 model as a powerful tool for investigating novel agents in colorectal cancer. Further research should explore SIRT2-regulated pathways in greater depth, assess combinatorial strategies with existing therapies, and expand findings to metastatic or orthotopic models to enhance translational impact.

Precision Immunotherapy Using CT26 and Engineered Bacterial Vectors

Programmable delivery of immune checkpoint inhibitors represents a transformative direction in immuno-oncology, particularly when applied to colon carcinoma models such as CT26. The CT26 cell line, derived from murine colorectal carcinoma, serves as a well-characterized platform for investigating immune evasion mechanisms and therapeutic resistance in microsatellite stable tumors. One of the critical immune escape pathways exploited by CT26 tumors involves the overexpression of CD47, a transmembrane protein that interacts with SIRPα on macrophages to inhibit phagocytosis. Disrupting this axis enhances macrophage-mediated clearance and facilitates antigen presentation to cytotoxic T cells. A promising approach involves using engineered bacterial vectors designed to respond to hypoxic tumor microenvironments by producing anti-CD47 antibodies in situ. When paired with liposomes carrying macrophage-activating agents such as M-CSF, this strategy promotes both macrophage infiltration and polarization toward an M1 phenotype, reinforcing the innate immune response.

In CT26-based models, this method has shown improved tumor penetration, enhanced recruitment of CD8+ T cells, and reduction in both primary tumor burden and metastatic lesions. Hypoxia-sensitive genetic circuits within the bacterial vectors enable spatiotemporal control of therapeutic antibody production, limiting systemic exposure and reducing toxicity. These engineered constructs can evade neutrophil clearance, accumulate in necrotic tumor regions, and produce immunologically active molecules directly within the tumor. Data indicate elevated expression of inflammatory markers, increased phagocytic activity, and greater T cell activation, culminating in robust antitumor immunity. Importantly, this localized approach to immune checkpoint blockade also results in modulation of the tumor-associated microbiome, potentially contributing to sustained therapeutic effects. Collectively, these findings underscore the relevance of the CT26 model in evaluating next-generation immunotherapies and suggest that precision delivery platforms may overcome key limitations associated with conventional systemic treatments. Further exploration in diverse tumor contexts and host immune backgrounds is warranted to fully define the translational potential of this approach.

Assessing Therapeutic Efficacy and Immune Interaction in CT26 Tumors

The CT26 murine model of colorectal cancer provides a valuable system for evaluating therapies that target oncogenic K-Ras mutations, especially the G12D variant, which is common in gastrointestinal tumors. A bicyclic peptide inhibitor developed for K-Ras(G12D) was found to suppress CT26 cell proliferation and colony formation in a dose-dependent manner, accompanied by reduced phosphorylation of ERK, a key signaling mediator downstream of Ras. Although the peptide requires micromolar concentrations to achieve biological effects, likely due to limited cell membrane permeability, it induces modest apoptosis and primarily restricts tumor growth through cytostatic mechanisms. *In vivo*, the compound significantly reduces tumor volume in CT26 allografts without causing weight loss or pathological changes in the liver or kidneys, indicating a favorable safety profile for further preclinical development.

However, when combined with an immune checkpoint inhibitor, no additive or synergistic effect was observed. Analysis of CT26 tumor samples showed no significant differences in CD8+ T cell infiltration or PD-L1 expression among treatment groups. Pharmacokinetic profiling revealed a short plasma half-life, strong plasma protein binding, and notable accumulation within blood cells, all of which may limit systemic availability and interfere with immunotherapeutic interactions. These findings underscore the importance of considering drug distribution, immune modulation, and delivery mechanisms when designing combination therapies. Further investigation using metastatic or orthotopic CT26 models, along with improved formulations or delivery platforms, will be essential to realize the full therapeutic potential of K-Ras(G12D)-targeting agents.

Immune Context Shapes Oncogenic Signaling in CT26 Tumors

The CT26 murine colon carcinoma model is valuable for studying the effects of immune context on tumor evolution, particularly the reprogramming of oncogenic signaling and immune recognition. In immunocompetent hosts, CT26 tumors typically grow rapidly, showing low antigenicity and moderate immune infiltration. However, when introduced into immunocompromised hosts such as NOD.SCID mice, early tumor growth slows markedly, despite the concurrent silencing of key tumor suppressors including PTEN and RBL1. Proteomic analysis of CT26 tumors revealed a significant reprogramming of intracellular signaling pathways in the absence of immune pressure, notably involving the PI3K/AKT/mTOR and TP53 networks. This shift appears to facilitate survival in a stressed tumor microenvironment while

simultaneously enhancing genomic instability. As tumors progress in this immunodeficient context, they begin to express elevated levels of tumor-associated antigens and become increasingly immunogenic.

Re-inoculation of these CT26 tumors into immunocompetent BALB/c mice further demonstrated their heightened immunogenic profile. Approximately 70 percent of the tumors originally grown in NOD.SCID mice regressed upon transfer, in contrast to near-complete progression observed with CT26 tumors derived from BALB/c hosts. This regression was accompanied by a robust infiltration of antigen-presenting cells and CD3+ T lymphocytes, indicating effective activation of adaptive immunity. The findings suggest that the absence of immune editing in immunodeficient hosts permits the accumulation of immunogenic tumor subclones with preserved or elevated antigen expression. This mechanism may underlie the paradoxical observation that tumors from immune-deficient environments can become more immunogenic over time. Furthermore, the data imply that stress-induced signaling alterations, including endoplasmic reticulum stress and deregulation of protein processing pathways, may act as co-factors in antigen emergence. These insights position CT26 as a powerful model for understanding immune-tumor coevolution, tumor antigenicity, and the design of strategies to enhance the efficacy of cancer immunotherapies through controlled modulation of the tumor microenvironment.

Advancing Immunotherapy with Syngeneic Mouse Models

Syngeneic mouse models represent a foundational platform in cancer research, particularly for elucidating interactions between tumor cells and the host immune system. These models are established by transplanting tumor cells into genetically identical, immunocompetent mice, thereby preserving the integrity of immune responses that would be absent in immunodeficient systems. Their capacity to replicate native immune-tumor dynamics makes them especially well-suited for preclinical evaluation of immunotherapies. Syngeneic models enable detailed investigation into immune evasion mechanisms, immune cell infiltration, and the functional consequences of therapeutic interventions on both innate and adaptive immune responses. Moreover, they provide a robust framework for studying metastatic dissemination, tumor microenvironmental modulation, and immune-mediated tumor clearance. By maintaining a fully functional immune system, these models yield insights that are highly relevant to the clinical development of immunomodulatory cancer therapies.

Animal care and handling at Altogen Labs are conducted in accordance with IACUC regulations and under GLP-compliant conditions, ensuring adherence to the highest standards of animal welfare and experimental reproducibility. Upon arrival and acclimatization to the vivarium environment, mice are sorted by body mass to minimize variability in tumor implantation outcomes. Daily assessments are performed to monitor tumor onset, general health, and clinical signs. These observations are critical for early detection of treatment-related toxicity and for maintaining consistency in tumor progression metrics. In addition to routine monitoring, the facility provides a comprehensive range of analytical services including tissue collection, histological examination, protein and RNA isolation, and gene expression profiling. These services enable detailed molecular and phenotypic characterization of therapeutic responses across various treatment cohorts.

Figure 4. Overview of *in vivo* allograft services for the CT26 murine colon carcinoma model at Altogen Labs, highlighting the range of available drug administration routes used for preclinical oncology studies.

Altogen Labs offers a wide array of customizable options for studies utilizing the CT26 syngeneic mouse model. Available endpoints include tumor growth delay (TGD), tumor growth inhibition (TGI), toxicity, survival, and blood chemistry analysis. Dosing parameters can be tailored by frequency, duration, and route of administration, including intravenous, intratracheal, continuous infusion, intraperitoneal, intratumoral, oral gavage, topical, subcutaneous, intramuscular, and intranasal delivery using advanced micro-injection and pump-controlled IV systems. This flexibility allows researchers to replicate a variety of clinical dosing scenarios and optimize pharmacokinetic parameters. Researchers can also select from alternative engraftment sites such as orthotopic transplantation, tail vein or left ventricular injection for metastasis models, and mammary fat pad or peritoneal cavity injection. Imaging capabilities include fluorescence-based whole-body imaging and magnetic resonance imaging (MRI), while immunohistochemical analysis of CT26 tumors supports detailed evaluation of immune cell infiltration, tumor vascularization, and therapeutic modulation of the tumor microenvironment.

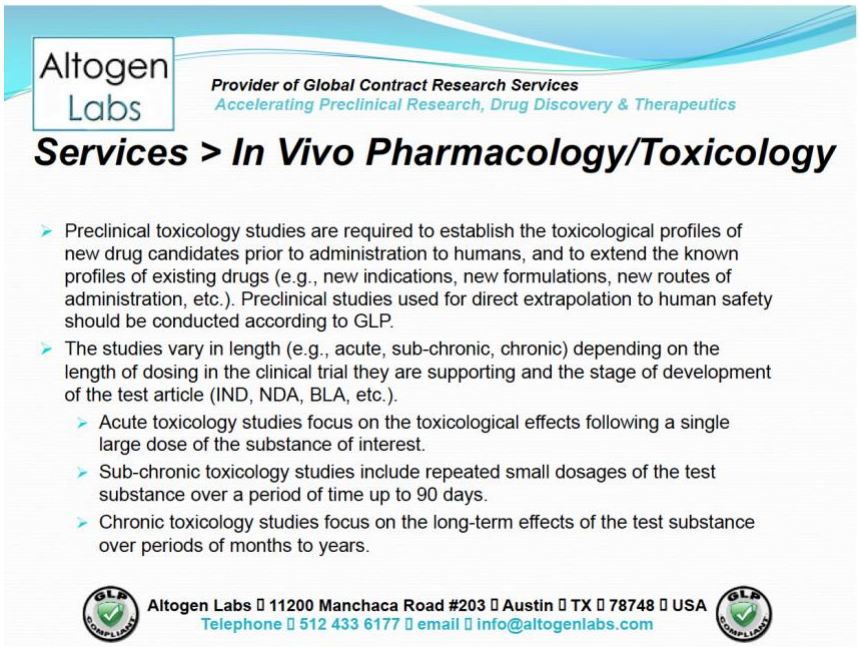

Figure 5. Summary of *in vivo* pharmacology and toxicology services offered by Altogen Labs, including acute, sub-chronic, and chronic toxicity study designs conducted in compliance with GLP to support preclinical safety evaluations of investigational compounds.

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