

Engineering A p53-Independent Therapeutic Switch: A Hypothetical Framework for AAV-Driven Expression of p21, Gadd45, and Bax for Controlled Cancer Cell Fate

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Abstract

Background: Gene therapy is a promising field in modern medicine, particularly for cancer treatment. Viral vectors, particularly Adeno-associated virus, provide efficient gene delivery and have advanced rapidly due to their advantages over other gene-carrying vectors, such as lack of pathogenicity, ability to transfect both dividing and non-dividing cells, low host immune response, and long-term expression.

Methods: The paper offers a synthetic gene circuit that can bypass defective p53 in cancer cells without directly addressing it. Using alternative genetic modules such as GADD45, p21, and BAX, the system reconstructs critical p53 capabilities such as cell cycle arrest, DNA repair, and apoptosis.

Results: This technique aims to use targeted gene therapy to achieve controlled and selective tumor suppression.

Conclusions: This synthetic gene circuit provides a strong therapeutic framework demonstrating that using different modules to get around p53-deficiency may be a practical route for targeted cancer treatment in the future.

Trial registration: Not applicable.

Keywords: Solid Tumors, Gene Therapy, Recombinant Adeno-Associated Virus (Raav), Synthetic Gene Circuit, P53-Deficiency, Bax, Gadd45, P21, Targeted Cancer Therapy

Introduction

DNA accumulation has a severe negative impact on human health, causing cellular senescence, ageing, apoptosis, and genome instability. This gradual damage contributes to the change of healthy cells into malignant ones. In cancer, cells' ability to respond to the DNA damage response (DDR) is essential, as inadequate repair of DNA damage may result in cellular demise [1-3].

DNA damage can take several forms, including single-strand breaks (SSBs), double-strand breaks (DSBs), base changes including oxidation and deamination, and massive lesions caused by UV radiation or chemical exposure. Cells use a number of repair processes to maintain genomic integrity, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break repair pathways such as homologous recombination (HR) and non-homologous end joining (NHEJ) [4].

Checkpoint systems, such as the G1/S, S, and G2/M checkpoints, control cell cycle progression by detecting DNA damage and

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halting the cycle to allow repair. The key proteins involved in this process include Tumor Protein p53 (p53), Ataxia-Telangiectasia Mutated (ATM), and Ataxia-Telangiectasia and Rad3-related (ATR). If the damage is irreparable, these pathways can cause apoptosis, halting the spread of abnormal cells [5].

The tumor suppressor protein p53 controls cell cycle arrest, DNA repair, and apoptosis. However, mutations in the TP53 gene can impair its ability to bind to DNA, preventing it from performing its regulatory functions. Furthermore, overexpression of negative regulators such as Mouse Double Minute 2 (MDM2) can promote p53 degradation, even in conditions where it is required. As a result, cells lose a critical barrier, allowing uncontrolled growth and cancer development [6].

Cyclin- dependent kinase (CDK) inhibitor 1A, or p21, is a member of the Cip/Kip family of CDK inhibitors, also known as p21 WAF1/Cip1 or CDK inhibitor 1A [7]. P21, a tumour suppressor gene, was discovered and plays a vital role in regulating cancer proliferation. p21 has several tasks, including inhibiting the cell cycle, inducing senescence, and regulating DNA repair and death. It inhibits cyclin-CDK complexes, particularly during the G1/S and G2/M transitions, preventing cell cycle progression and allowing for DNA repair [8, 9].

The Growth Arrest and DNA Damage-inducible 45 (GADD45) protein family plays a role in cellular stress responses, activating fast response to DNA damage and oncogenic stress. They participate in DNA repair, cell cycle regulation, and apoptosis. GADD45 proteins interact with crucial regulators like proliferating cell nuclear antigen (PCNA) and p21, and they inhibit CDK1 activity, which is essential for controlling the G2/M transition [10].

Apoptosis is a controlled process that kills damaged or diseased cells via two main pathways: extrinsic and intrinsic. The Bcl-2 family regulates the intrinsic apoptotic pathway and comprises both anti-apoptotic and pro-apoptotic proteins. BAX is a pro-apoptotic gene that induces apoptosis through mitochondrial processes [11].

Based on these mechanisms, targeting important regulators of cell cycle progression, DNA repair, and apoptosis, such as p21, GADD45, and BAX, may be a gene therapy strategy to compensate for p53 dysfunction and increase cancer cell elimination [12].

Cell cycle arrest, DNA repair, and apoptosis can help to compensate for cancer cells' loss of functioning p53 signalling. Rather than simply replacing the natural p53 protein, our method aims to rebuild a minimum functional module that captures the most therapeutically relevant downstream effects. The coordinated and context-dependent co-expression of the genetic trio p21, GADD45, and BAX is postulated as a partial reconstitution of the p53 network's critical regulatory outputs. Through regulated activation, p21 is intended to cause cell cycle arrest, GADD45 to boost DNA repair pathways, and BAX to begin mitochondrial-mediated apoptosis in a tumor-specific fashion. This constructed circuit intends to restore homeostatic regulation within cancer cells with damaged or altered p53 pathways, thereby inhibiting tumour progression. Importantly,

this method does not attempt to mimic the complexity of natural p53 signaling entirely, but rather seeks to compensate for its key tumour suppressive effects functionally. This technique creates a modular and potentially customisable therapeutic framework that can act independently of upstream p53 failure while staying responsive to the cellular milieu. As a result, this approach may provide a unique avenue for targeted cancer therapy, particularly in solid tumors displaying high rates of p53 mutations and resistance to conventional therapies. Activation of this circuit may be further refined through tumor-specific triggers, such as hypoxia-responsive elements or aberrant metabolic signals, to enhance selectivity and minimize off-target effects.

Key Molecular Target

Growth Arrest and DNA Damage-Inducible 45 (GADD45) Family

Overview and Functions in Cells

The GADD45 proteins (alpha, beta, and gamma) are critical for maintaining genomic stability and play significant roles in the physiological stress response. The DDR, a complex network of tolerance mechanisms, repair pathways, and cell cycle checkpoints that detect and repair DNA damage, is principally responsible for GADD45 tasks [13].

Transcriptional and Post-Transcriptional Regulation

GADD45 gene expression is tightly regulated at both the transcriptional and post-transcriptional levels in response to cellular stressors such as DNA damage, UV radiation, oxidative stress, and ionising radiation. Transcriptional Level: GADD45 genes are in a "poised" chromatin state, which allows for rapid activation in response to stress with minimal chromatin remodelling. GADD45 induction is associated with DNA damage and oxidative stress responses via important transcription factors such as p53, BRCA1, and FOXO family members (FOXO3a, FOXO4).

MAPK cascades (especially the MTK1 / MEKK4 - p38 - JNK axis) and ERK signalling can also modulate GADD45 alpha expression after chemotherapy [14]. RNA-binding proteins including AU-rich element RNA-binding protein 1 (AUF1) and TIA-1-related protein (TIAR) affect the stability of GADD45 mRNA by binding to the 3'UTR and inducing mRNA degradation under non-stressed conditions, thereby maintaining low baseline expression. In contrast, GADD45 alpha can stabilise specific target mRNAs, adding a regulatory layer to stress adaptation [15].

Functions Specific to Isoforms and Their Contexts

GADD45 regulation is highly context-dependent, with different isoforms playing distinct functions. GADD45 gamma is typically suppressed in tumors by promoter methylation. GADD45 alpha is frequently overexpressed in certain tumours (e.g., pancreatic cancer), where it can either promote apoptosis or boost DNA repair and survival, depending on the signalling context. GADD45 beta: It plays dual roles in survival and tumour suppression through pathways such as constitutive androstane receptor (CAR) signalling and p38 MAPK activation [10].

Therapeutic Relevance

Overall, GADD45 functions as a stress-responsive molecular hub, integrating DNA damage signals, epigenetic state, and

kinase signalling pathways to provide tightly controlled and context-specific cellular outcomes such as repair, apoptosis, tumour suppression, and survival. As a result, gene therapy options, including Adeno-associated virus (AAV) as a delivery route, are being investigated to restore GADD45 function in tumours where it has been lost. Furthermore, tumor-selective delivery techniques are required to ensure that GADD45 restoration happens preferentially in cancer cells while having minimal impact on normal tissues, hence increasing therapeutic specificity and safety [10].

p21 (CDKN1A)

Structural Domain Characterisation

p21 is a 164-amino-acid protein that has a cyclin-dependent kinase inhibitor domain. The N-terminal region contains cyclin and CDK binding motifs. In contrast, the C-terminal region contains a nuclear localisation signal as well as binding sites for the replication protein PCNA and poly (ADP-ribose) [16].

Position in Cancer Dynamics and Dual Functions

The role of p21 in many cancer types has been thoroughly investigated utilizing cell lines, primary cultures, and patient samples. Tumour Suppressive Effects: Stabilizing p21 at both the mRNA and protein levels causes cell cycle arrest in cancer cells [16]. Oncogenic and pro-survival effects: Hypermethylation of the p21 promoter and the Binding of other regulatory proteins, such as SRY-box transcription factor 2 (SOX2) and lamin B2 (LMNB2), causes cancer cell proliferation by suppressing their expression. Conversely, p21 has been shown to promote cancer growth by stabilising Cyclin D1-CDK4 complexes and preventing apoptosis [16,17]. Thus, precise modulation of p21 expression levels and duration is required in therapeutic situations to assure tumor-suppressive effects while avoiding potential pro-survival or carcinogenic implications [18].

In Vivo Knockout Phenotypes and Mutational Burden

In mouse models, p21 deletion does not affect carcinogenesis until about 16 months, at which point spontaneous tumours appear. Other characteristics observed in p21 knockout mice include developmental defects such as larger spleens, hearts, and smaller testes. The mutational burden of CDKN1A mutations varies depending on the kind of mutation and the cancer type. Overall, p21 is altered in 0.96% of solid tumour cancers. Among these, bladder cancer has the highest rate of mutations within p21, affecting 11.99% of patients [16,19].

Cell Cycle Arrest and DNA Replication

Stress in the DDR: Cell cycle arrest is critical during the DDR, making p21 an essential inhibitor in this process. A study combining time-lapse photography and Single-cell tracking found that, after injury, p21 levels must rise over a threshold before the cell enters the S phase, which stops the cell cycle [20]. Interaction with Translesion DNA Synthesis (TLS): A noteworthy exception is TLS, which incorporates polymerase η (pol η) and binds to PCNA. In the absence of replication stress, p21 binds to PCNA, preventing it from engaging with pol η and so blocking TLS. Furthermore, following DNA damage induced by UV, methyl methane sulfonate, or hydroxyurea, p21 levels fall, and TLS takes place [1]. S-Phase Degradation and Senescence: Single-cell studies have shown that following an

initial rise via the p53 pathway, p21 is actively destroyed during the S phase to prevent genomic instability, as excessive levels of p21 hinder DNA repair. When p21 levels remain high, the cell may undergo senescence or apoptosis. Notably, continuous p21 expression in the absence of p53 results in a subpopulation that re-enters the cell cycle, a condition known as re-replication stress, which can cause genomic instability [21].

BAX (Bcl-2-Associated X Protein)

Mechanisms for Mitochondrial Apoptosis

The activation of BAX and BAK is the final step in intrinsic (mitochondrial) apoptosis, leading to mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, and caspase activation. Their oligomerisation creates holes in the mitochondrial membrane, resulting in an irreversible commitment to cell death [11]. Stress signals are incorporated upstream to control this activation, making BAX/BAK the primary decision nodes for mitochondrial apoptosis [11].

Sublethal Activation and Oncogenic Risk

However, in some cells, the caspase cascade might be initiated partially or sublethally, resulting in incomplete apoptosis and DNA damage caused by caspase-activated DNase. This incomplete activation may raise mutation rates and perhaps promote tumour formation, emphasising the importance of fully and irreversibly activating caspases for effective therapeutic apoptosis [22].

Implications of Gene Therapy

Overall, BAX/BAK activation is the irreversible commitment stage in mitochondrial apoptosis, making it an important downstream effector for numerous treatment methods. In the context of gene therapy-based cancer treatments, therapeutic design can focus on restoring or improving pro-apoptotic signalling upstream of the checkpoint. For example, releasing or reactivating genes that enhance cellular stress response signalling or DNA damage sensing might indirectly induce BAX/BAK activation and mitochondrial death in tumour cells [23]. This technique aims to overcome cancer's resistance to apoptosis by restoring appropriate intrinsic death signalling, guaranteeing that when DNA damage or oncogenic stress is identified, cells efficiently proceed to full caspase activation and regulated cell death. As a result, targeting pathways that converge on BAX/BAK activation provides a reasonable foundation for gene therapy techniques aimed at sensitising tumours and increasing treatment efficacy while minimising partial or incomplete apoptotic signaling [24].

Functional Interaction of GADD45, p21 and BAX

In apoptosis, the interplay of GADD45, p21, and BAX forms a coordinated regulatory network that connects DNA damage sensing, cell cycle control, and apoptotic execution. GADD45 functions as an upstream stress sensor, responding quickly to DNA damage and activation of signalling pathways, including p38 MAPK and JNK [25,26]. Through these signalling mechanisms, GADD45 coordinates with p21 to facilitate cell cycle arrest at crucial checkpoints. This arrest enables cells to assess DNA damage and choose between repair and apoptosis. In this setting, p21 acts as a context-dependent regulatory checkpoint protein, determining cellular destiny. While transitory p21 activation

promotes DNA repair and survival, prolonged or dysregulated expression, particularly in the absence of functioning p53, can tip the balance toward apoptosis or senescence [12, 27, 28]. Under conditions of severe or irreversible DNA damage, signalling cascades initiated by GADD45 and regulated by p21 eventually lead to the activation of BAX. BAX is conformationally activated and translocates to the mitochondrial outer membrane, where it promotes MOMP, resulting in cytochrome C release and caspase activation [11]. Thus, GADD45, p21, and BAX serve as a sequential and interrelated axis. GADD45 is involved in damage sensing and signalling, while p21 is involved in cell cycle arrest and fate choice, and BAX is responsible for irreversible apoptotic execution. This coordinated interaction provides a mechanistic framework for gene therapy strategies aimed at restoring tumor suppressive pathways in p53-deficient cancers (Figure 1) [11, 12, 25].

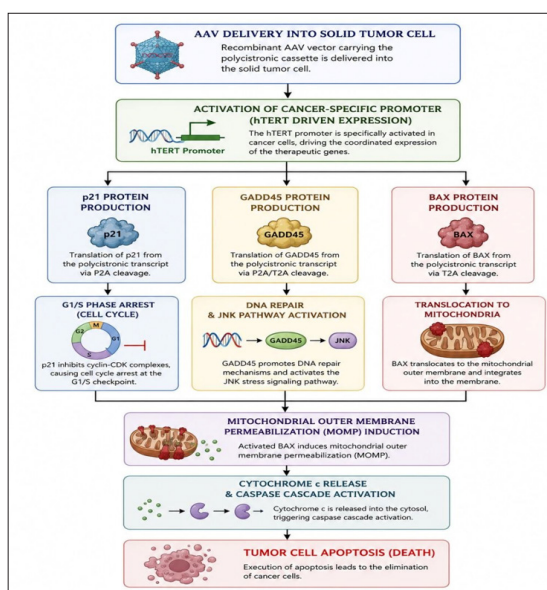


Figure 1: Schematic representation of the proposed synthetic gene circuit. The diagram illustrates the delivery of the AAV vector into the solid tumor cell, the hTERT-driven expression of p21, GADD45, and BAX, and the subsequent activation of cell cycle arrest, DNA repair, and mitochondrial apoptosis pathways, ultimately leading to tumor cell elimination.

Problem Statement:

Cellular regulatory networks and malignancy

Human tumours progress due to the failure of networks controlling cell cycle checkpoints, DDR, and intrinsic apoptosis.

Upstream Pathway Dysregulation

In many cancers, abnormal p53 signalling impairs transcriptional activation of the expression of downstream effectors like CDKN1A (p21) and the GADD45 family, thereby enabling cells with compromised genomic integrity to bypass replicative senescence [9].

Apoptotic Suppression and Treatment Resistance

Suppressing MOMP by inactivating or downregulating BAX/BAK provides a survival benefit and promotes multidrug resistance (MDR) in solid tumours [29, 30].

The Limits of Conventional Therapeutics

Standard cytotoxic regimens, including genotoxic chemotherapies and ionising radiation, try to exploit these vulnerabilities. However, their clinical success is limited by a lack of cellular selectivity and rapid selection of resistant clones [31].

Current Therapeutic Gaps

Contemporary oncological treatments lack a multi-pronged intervention that can restore cell cycle arrest, modulate DNA repair dynamics, and reactivate downstream apoptotic cascades in a cohesive delivery platform.

Proposed Coordinated Gene Therapy Approach

To overcome treatment constraints, we propose using adeno-associated virus (AAV) vectors [32] to restore p21 and GADD45 expression while promoting BAX-mediated apoptosis in solid malignancies.

Materials and Methods

The Rationale for Vector Selection and Packaging Constraints

Genes will be delivered using a genetically designed AAV vector. The rationale for this pick is based on AAV's better transduction efficiency as well as its capacity to infect both dividing and non-dividing (quiescent) human cells. To maximise clinical safety and ensure precise temporal control over transgenic expression, the vector will be designed to be replication defective. This design must account for AAV's low packaging capacity of 4.7-5.0 kb. Because our technique enables the simultaneous delivery of three separate therapeutic genes, CDKN1A (p21), GADD45, and BAX, the expression cassette is constructed as a polycistronic vector including 2A self-cleaving peptides (such as P2A or T2A) or Internal Ribosome Entry Site (IRES) elements. This configuration ensures the stoichiometric expression of all three downstream effectors from a single transcript without exceeding the physical packaging constraints of the viral capsid [33]. Additionally, inverted terminal repeats (ITRs) are located at the genomic flanks and serve as essential structural elements that drive viral DNA replication, rescue, and encapsulation [34].

AAV Infection Mechanism: Cell Attachment, Entry, and Nuclear Transport

Cellular entrance includes a sequence of receptor-mediated interactions:

Cellular Attachment

The initial binding event takes place when the main attachment receptors on the host Cell surface connect, primarily Heparan Sulphate Proteoglycans (HSPG). Following high-affinity engagement with secondary membrane co-receptors such as Fibroblast Growth Factor Receptor 1 (FGFR1), an essential receptor AAV Receptor (AAVR), the viral particle is endocytosed via clathrin [35].

Endosomal Entry and Cytoplasmic Escape

Following endocytic engulfment, the gradual acidification of the endosomal compartment causes critical conformational changes in the viral capsid proteins (specifically, exposing the VP1/VP2 phospholipase A2 domain), allowing endosomal membrane disruption and subsequent escape into the cytoplasm [36]. The virus travels retrogradely through the host cell's

microtubule network using dynein motors, eventually reaching the nuclear pore complex. After passing through the pore into the nucleoplasm, the viral capsid uncoils, allowing the single-stranded DNA genome to undergo second-strand synthesis and begins gene transcription [37].

Addressing Existing Therapeutic Challenges

This project aims to address two major barriers for AAV-based delivery:

Pre-existing Immunity and Immunogenicity

Pre-existing neutralising antibodies against common human AAV serotypes are found in 40-80% of the population, which can dramatically reduce vector effectiveness [38]. To overcome this, a two-stage immunisation technique will be used:

Initial Dose: We will use designed non-human AAV serotypes (e.g., bovine AAV) or antigenically different variations that patients have not previously encountered [39].

To minimise secondary memory responses, sequential dosing can be handled through heterologous boosting with a different serotype. This also helps to prime tumour antigen-specific vectors [40]. (e.g., chimpanzee AAV or other unusual animal AAVs).

Tissue Targeting and Tropism Specificity

Controlling the biodistribution of AAV vectors is critical to minimise off-target effects. Tissue targeting will be refined through several rational design approaches:

Epigenetic Status and Genomic Integration: While recombinant AAV vectors lack the rep gene and typically persist episomally within the nucleus as stable, non-integrating circular concatemers, thereby eliminating the risk of insertional mutations [33], specific rational designs can target wild-type integration mechanics at the chromosome 19 AAVS1 locus if long-term, lineage-specific genomic permanence is required.

Small Molecule Inducible Systems: Temporal control of gene expression will be managed via inducible systems. At the transcriptional level, we can implement Tet-on/off (doxycycline-inducible) or rapamycin-inducible systems. Post-transcriptionally, the inclusion of riboswitches, aptazymes, or miRNA-responsive elements can fine-tune RNA stability. Furthermore, post-translational regulation can be achieved via degron systems to selectively target proteins for degradation [41-43].

Rational Design Solution

To provide accurate tissue specificity, four critical engineering solutions will be combined:

Capsid Engineering for Evasion: Using mutation techniques to replace surface tyrosine residues with phenylalanine. Because tyrosine is an important target for ubiquitination-mediated destruction, these alterations increase capsid stability and transduction efficiency [33].

Targeted promoters, like the human telomerase reverse transcriptase (hTERT) promoter, have been utilized to achieve

tumor-selective expression of therapeutic genes in cancer gene therapy [44].

Receptor Binding Modification: Engineering capsid surface areas to alter receptor tropism. For example, modified AAV2 capsids reduced binding to heparin sulphate, allowing for deeper tissue penetration [44].

Tissue-Specific Ligand Conjugation: Targeting specific organs, like the liver, with GalNAc (N-Acetylgalactosamine)-conjugated vectors [33].

Transgene Design and Delivery Methods

Expression Cassette Architecture

To achieve coordinated expression within a single vector, the multi-cistronic expression cassette will be structured sequentially: [ITR] -- hTERT Promoter -- p21 -- P2A -- GADD45 -- T2A -- BAX -- PolyA -- [ITR].

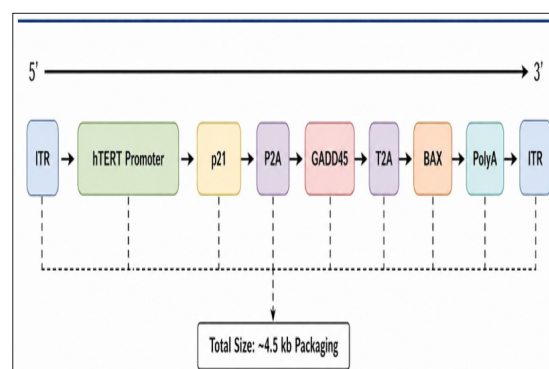


Figure 2: Schematic representation of the polycistronic AAV expression cassette

The therapeutic cassette is flanked by inverted terminal repeats (ITRs) required for viral replication and packaging. The hTERT promoter drives tumor-specific expression of the polycistronic transcript, encoding p21, GADD45, and BAX separated by self-cleaving 2A peptide sequences (P2A and T2A) to ensure stoichiometric expression of each gene. A polyadenylation signal (PolyA) at the 3' end enhances transcript stability and translation efficiency. The total size of the cassette is approximately 4.5 kb, which falls within the AAV packaging capacity limit (~4.7–5.0 kb).

The downstream arrangement of the transgenes [hTERT-p21-P2A-GADD45-T2A- BAX-PolyA] is rationally planned using translational stoichiometry. Positioning the highly potent pro-apoptotic effector BAX at the terminal 3' is an intentional regulation technique. Downstream cistrons after several 2A-skipping sites usually show calibrated translational attenuation (Figure 2) [45]. This strategy prioritises strong initial expression of p21 and GADD45 to impose cell cycle checkpoints and activate DNA repair machinery initially, while minimizing the risk of premature or excessive BAX-mediated cytotoxicity during viral vector packing and production in host packaging cells.

Routes of Administration

Two strategic delivery options are assessed based on illness burden:

Systemic Delivery ("In Vivo"): Used intravenously to treat metastatic or extensive tumours, ensuring vector access to distal tumours.

Localized delivery focuses on accessible primary solid tumours, reducing off-target exposure.

The choice of delivery method entails important trade-offs between widespread viral dissemination efficiency and local targeting precision.

Experimental Design and Validation

In Vitro Malignancy Studies

The modified gene circuit will be validated using existing p53-deficient cancer cell lines to assess its therapeutic impact in a controlled environment. These cell types will be transduced using an engineered recombinant AAV (rAAV) vector containing the polycistronic therapeutic cassette.

After successful viral transduction, cellular responses will be measured using three major axes:

Cell viability assays measure the intervention's overall cytotoxic and anti-proliferative effects and efficacy.

Apoptosis markers are used to confirm that transgene expression activates programmed cell death pathways.

DNA Damage and Repair Markers: Monitor changes in genomic stress profile caused by the genetic circuit.

In Vivo Preclinical Studies

To establish therapeutic efficacy in complicated physiological environments, a preclinical study will move to tumour-bearing animal models. These *in vivo* assays will be used to track systemic and localized responses over a predetermined time period. The main goals of these animal studies will include a careful examination of tumour size reduction trends as well as overall animal survival rates.

Analytical Methodologies and Advanced Assays

Specialized molecular and cellular assays will be conducted to determine accurately the downstream effects of gene therapy:

Cell Cycle Progression Analysis: Flow cytometry will analyse cell cycle and discover p21-mediated G1/S phase arrest.

DNA Repair Efficiency Assessment: The functional activation of DNA repair mechanisms and genomic integrity shifts will be measured by gamma-H2AX immunofluorescence and comet tests.

Cell Fate Outcome Evaluation: We will assess apoptosis levels to determine the impact of activating the BAX pathway on cell fate.

Quantitative Polymerase Chain Reaction (qPCR) and Western blotting will be used simultaneously to confirm and quantify transgenic expression at the mRNA and protein levels.

Furthermore, to assess the polycistronic transcript's ribosomal skipping efficiency, Western blotting techniques will use particular antibodies directed against the residual 2A peptide

'tails' (such as the C-terminal amino acid fragments left on p21 and GADD45). This accurate measurement is required to demonstrate full peptide cleavage and rule out the buildup of abnormal, uncleaved fusion proteins, which could jeopardise the therapeutic triad's native tertiary structure or functional potency [45].

Expected Results

In Vitro Stoichiometric Transgene Expression and Cytotoxicity
After transducing p53-deficient solid tumour cell lines with the polycistronic rAAV Vector, we expect to confirm synchronised mRNA and protein expression for p21, GADD45, and BAX using qPCR and Western blot analysis. Mechanistically, flow cytometry analysis of the cell cycle is predicted to demonstrate a considerable, threshold-transcending increase of cells stalled at the G1/S transition, which is directly driven by p21-mediated cyclin-CDK inhibition [7]. Simultaneously, immunofluorescence for gamma-H2AX and comet tests should confirm that GADD45 Expression successfully regulates DNA repair kinetics during genomic stress [10]. Finally, cell viability experiments are predicted to show a significant reduction in tumour cell growth, leading to irreversible, full-scale apoptosis execution as evidenced by strong BAX activation levels [11].

In Vivo Tumour Regression and Survival Kinetics

The rAAV vector is projected to have high therapeutic efficacy in preclinical tumor-bearing animal models, both locally and systemically administered. We predict a robust, time-dependent restriction of solid tumour volume growth, which longitudinal physical measurements will assess. Histopathological examination of removed tumour tissues should reveal widespread necrotic and apoptotic zones that correlate with viral transduction targets. Furthermore, animals treated with the triple-gene circuit are projected to have higher long-term survival rates than control groups, with no systemic phenotypic toxicities or significant off-target structural damage.

Limitations

Vector Packaging Overload and Internal Cleaving Kinetics

The use of 2A self-cleaving peptides (P2A/T2A) allows for numerous cistrons to be expressed from a single transcript; however, the payload size exceeds the AAV capsid's 5.0 kb packing limit [45]. This close closeness to full capacity may diminish viral packaging efficiency, lower total viral titers throughout production, or result in partial synthesis of the entire polycistronic genome [46]. Furthermore, any baseline inefficiency in the ribosomal skipping mechanism of the P2A or T2A peptides may result in the generation of abnormal, uncleaved fusion proteins, thereby jeopardizing the native tertiary structure and functional potency of p21, GADD45, or BAX [46].

To address this concern, a detailed theoretical quantification of the cargo Quantity was carried out. The concatenated coding sequences (CDS) for CDKN1A (~500 bp), GADD45a (~500 bp) and BAX (~600 bp) produce a structural payload of around 1.6 kb. Even after accounting for cis-regulatory factors such as the tumor-restricted hTERT promoter, 2A self-cleaving linker sequences, a synthetic polyadenylation signal, and flanking inverted terminal repeats (ITRs), the whole designed expression cassette measures between 3.8 and 4.2 kb. This is comfortably below the absolute wild-type AAV packing threshold of 4.7 to

5.0 kb, retaining viral titers and structural integrity during rescue and encapsulation.

Transduction efficiency in hypoxic solid tumour cores

Solid tumours have chaotic vascularization, high interstitial fluid pressure, and dense Extracellular matrices that hinder passive viral diffusion [47]. As a result, delivering rAAV vectors efficiently into the deeply buried, hypoxic core of a solid tumour remains a significant physiological challenge. While capsid alterations such as tyrosine-To-phenylalanine mutations improve stability; they may not completely overcome the anatomical hurdles posed by large solid tumour masses, potentially leaving untransduced cancer cell subpopulations capable of promoting tumour recurrence [33].

Future Perspectives

Integration of Tumour-Microenvironment Response Elements

Future gene circuits could include bidirectional, microenvironment-responsive Promoters for improved biosafety and therapeutic specificity. Engineering the expression cassette to be driven by hypoxia-responsive elements (HREs) or promoters that only respond to high lactate concentrations would limit transgene expression to the malignant core of solid tumours, virtually eliminating the risk of off-target toxicity in healthy, normoxic tissues.

Combinatorial Regimens for Immune Checkpoint Blockade

This gene therapy strategy has the potential to transform cold tumours into immunologically hot environments due to the release of tumour-associated antigens (TAAs) and damage-associated molecular patterns (DAMPs) during viral transduction and tumour cell lysis via BAX-mediated apoptosis. Future research should examine the synergistic therapeutic effects of combining this rAAV-mediated triple-gene intervention with systemic immune checkpoint inhibitors, such as anti-PD-1 or anti-CTLA-4 therapy, to stimulate systemic immunological memory and eradicate distant metastatic lesions.

Conclusion

Restoring Homeostasis via Synthetic Complementation

This study proposes a synthetic gene circuit to overcome dysregulation of the upstream p53 pathway in solid tumours. By delivering p21, GADD45, and BAX concurrently within a replication-deficient rAAV vector, the method successfully decouples critical downstream cellular fate checkpoints from mutant or inactive native transcriptional machinery. The sequential orchestration of cell cycle arrest, genomic stress regulation, and intrinsic apoptotic execution effectively generates a therapeutic switch capable of inducing targeted annihilation in cancer cells.

Clinical Prospects of the Axis Strategy.

The p21-GADD45-BAX axis offers a complex cancer platform, despite manufacturing and delivery hurdles arising from physiological constraints and viral packaging limitations. This method effectively overcomes the clinical limitations associated with traditional single-gene therapies and non-specific cytotoxic treatments. Finally, this coordinated genetic intervention represents a highly promising, mathematically structured paradigm change for attaining targeted tumour suppression in complex solid cancers.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests: The author declares that they have no competing interests.

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Authors' contributions: N.H. conceived the study, performed the design framework, and wrote the manuscript.

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