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Selective Thymic Glucocorticoid Receptor Modulation by Giving Pro-Drug Thymosine α-1 Immuno-Modulator in Chronic Level of Glucocorticoids Exposure Thus Preventing Autoreactive T Cells Production and Establishing Central Tolerance

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Abstract

Background: *We developing a pro-drug to protect the thymus against the atrophy caused by chronic glucocorticoids level in body and suppress production of auto reactive T cell from thymus without inhibiting normal T cell proliferation and thus prevent autoimmune disorder and delay various stages of autoimmunity.*

Conceptual Innovation and Scientific Rationale: *Re-engineering thymosin α-1 as a pro drug that protects T cell from chronic glucocorticoid levels. Thymosin α-1 potentiates T cell mediated immune response via differentiation and maturation of T cell progenitor cells3. Vamorolone (VBP 15) shows potent inhibition of pro inflammatory NF-kB(Nuclear Factor kappa light chain enhancer of activated B cells) pathways via high-affinity binding to the glucocorticoid receptor4.*

Proposed Concept and Methodology: *Create a pro drug by chemically conjugating thymosin α-1 peptide to a known SEGRM Vamorolone via a cleavable linker, where in thymus by cleaving through thymus specific serine protease it release thymosin α-1 which exerts its direct anti-apoptotic and immunomodulatory effects on thymocytes and simultaneously localized SEGRM Vamorolone modulates the glucocorticoid receptors to protect the stromal microenvironment from chronic glucocorticoids level and reduce inappropriate thymocytes death.*

Conclusion and Healthcare Implications: *Re-engineer Thymosin α-1 Pro drug thus helps thymocytes to build resistance from chronic high glucocorticoids level in body and maintain central tolerance without suppressing normal T cell proliferation. By protecting the thymus's ability to enforce central tolerance, this strategy aims to prevent the initial emergence of pathogenic autoreactive T cells and thus prevent auto-immune disorders.*

1. Background and Rationale

The thymus is the central organ involved in T cell development and production of naïve T cells. Systematic immuno-stimulation induces glucocorticoid mediated thymic involution with elevated serum glucocorticoid level and a diminishment of naïve T cells in the periphery [1]. Autoreactive T cells that escape from thymic elimination are activated by self-antigen can trigger auto immune response [2]. There is no drug proven in clinical studies to selectively bind and modulate thymic glucocorticoid receptor(GR) specifically to protect the thymus against the atrophy caused by high and chronic glucocorticoids level in body and suppress production of auto reactive T cell from thymus without inhibiting normal T cell proliferation.

1.1 Conceptual Innovation

Re-engineering thymosin α-1, a natural thymic peptide, as a pro drug that protects T cell from chronic glucocorticoid levels by modulating the glucocorticoid receptors that are located in

thymocytes requires conjugating thymosin α-1 with selective glucocorticoids receptor modulator(SEGRM) Vamorolone. Thymosin α-1 potentiates T cell mediated immune response via differentiation and maturation of T cell progenitor cells and action in elevating the activity of T cell maturation into CD4+/CD8+ T cells [3]. Vamorolone (VBP 15) is a first in class steroidal multifunctional drug that shows potent inhibition of pro inflammatory NF-kB(Nuclear Factor kappa light chain enhancer of activated B cells) pathways via high-affinity binding to the glucocorticoid receptor [4]. Thymus specific serine protease (TSSP) is a novel protease found in the thymus [5]. It is used to cleave re-engineered pro drug thymosin α-1 having thymosin α-1 conjugated with Vamorolone.

1.2 Scientific Rationale

It helps to re-establish central tolerance by promoting crucial process in the thymus that eliminates self-reactive T-Cells and generates regulatory T-cells. Thymosin α-1 is a peptide naturally occurring in the thymus that has long been recognised for modifying, enhancing, and restoring immune function. Thymosin α-1 has been utilised in the treatment of immunocompromised states and malignancies, as an enhances of vaccine responses [4]. Development of selective glucocorticoid receptors agonist and regulatory modulator (SEGGRAMs) aimed to minimise the adverse effects of chronic glucocorticoid treatments (Example-hyperglycemia and Osteoporosis) by separating the transactivation and transrepression activities of glucocorticoid receptors(GR) [6]. This proposal would prove that a Selective Glucocorticoid Receptor Modulators(SEGRM) can be engineered to selectively block GR mediated apoptosis and stromal damage within the thymus. There is no published research on designing a linker peptide that is selectively cleaved by Thymus Specific Serine Protease(TSSP) for drug delivery purposes.

2. Methodology

• Create a pro-drug by chemically conjugating Thymosin α-1 peptide to a known SEGRM(Selective glucocorticoid receptor modulator)
Vamorolone via a cleavable linker.
Design Objective:

Enzyme in Thymus
↓
Tα1 — Linker — [Cleavable Site] — Drug
↓
Drug Released

* Deliver Vamorolone selectively to thymic microenvironment
* Use Thymosin-α1 (Tα1) as targeting/immunomodulatory moiety
* Insert a linker cleavable by thymic serine proteases
* Release free Vamorolone upon enzymatic cleavage

2.1 Molecular Architecture:

Design:

[Thymosin α1]
|
Cysteine
|
Thioether (S)
|
Flexible Spacer
|
Serine-Protease Cleavable Peptide
|
PABC Self-Immolative Unit
|
Carbamate/Ester
|
[Vamorolone]

Steps:
Step A — Modify Thymosin α1
Provides: • Free thiol (–SH) • Site-specific maleimide conjugation
Step B — Design of Cleavable Linker
Linker components (in order):
Maleimide — Spacer — Protease-cleavable peptide — PABC (self-immolative spacer) — Activated ester — Vamorolone
Where:
Maleimide → reacts with Cys of Tα1
Peptide sequence → cleaved by serine protease
PABC spacer (para-aminobenzyl carbamate) → ensures clean drug release Vamorolone attached via carbamate/ester bond

• Physiological Journey of Pro Drug

Stage	Event	Key Requirement
Plasma	Stability	No premature cleavage
Distribution	Vascular delivery	Adequate perfusion
Thymus entry	Partial BTB crossing	Access to medulla
Cleavage	Serine protease activation	Specific linker
Release	PABC collapse	Clean drug liberation
Action	GR binding	Biased modulation

• Injection → Systemic Circulation
Route: IV (most direct) or SC (Sub-Cutaneous)
Key processes:- Plasma protein interaction with pro drug (albumin binding to large extent)

• Systemic Distribution Phase
Because the conjugate prodrug:
• Has increased molecular weight
• Is peptide-based
• Is hydrophilic It will:
• Distribute preferentially to highly vascular tissues The thymus is:
• Moderately vascular
• Supplied by branches of internal thoracic artery Drug reaches thymus via normal systemic perfusion.

• Vascular Entry into Thymus
Inside thymus
Blood enters through:
• Corticomedullary junction vessels Large molecules:
• Do NOT freely cross cortical BTB
• But can access:
o Medullary regions
o Perivascular spaces

conjugate pro drug likely reaches:
✓ Medullary thymic microenvironment
✓ Perivascular thymocyte niches

• Targeting Role of Thymosin α-1
Thymosin α-1 component:
• Has affinity for lymphoid tissue
• Interacts with immune signaling pathways

• Protease Recognition in Thymus
Inside thymic micro environment:

Relevant enzymes:
• Thymus-specific serine protease (TSSP)
This is the activation step.

• Self-Immolative Drug Release
After peptide cleavage
Free Vamorolone and Thymosin alpha 1 released Residual peptide fragment detached
Now drug becomes pharmacologically active.

• Thymosin alpha-1 – Mechanism After Release in Thymus
After protease-mediated cleavage of your prodrug construct, free Thymosin α1 (Tα1) acts on thymocytes and thymic epithelial cells through membrane-associated receptors, activating cAMP-dependent and PKC-dependent signaling pathways.

Protease cleavage → Free Thymosin α1
↓
Receptor binding
↓

Gs activation	Gq activation
↓	↓
Adenylyl cyclase	PLC activation
↓	↓
↑ cAMP	IP3 + DAG formation
↓	↓
PKA	PKC
↓	↓
CREB phosphorylation	NF-κB activation
↓	↓
↑ Bcl-2 expression	↑ IL-2R, MHC, cytokines
↓	↓
↓ Apoptosis	↑ Thymocyte maturation

• Vamorolone Ligand Doping and Dynamics on Glucocorticoid Receptors and Dynamics

Obtain GR-LBD Structure (RCSB PDB)
↓
Remove Native Ligand + Clean Protein
↓
Prepare Vamorolone Structure (Geometry Optimization, pH 7.4)
↓
MOLECULAR DOCKING (DOPING)

2.2 Define Steroid Binding Pocket

Flexible Ligand / Rigid Receptor Single Focused Docking Run
↓
Select Best Binding Pose
(Binding Energy + Interaction Map)
↓
Analyze Key Interactions:
• Hydrogen bonds
• Hydrophobic packing
• Helix-12 proximity
↓
MOLECULAR DYNAMICS SIMULATION
• Build GR–Vamorolone Complex
Solvate (TIP3P Water + 0.15 M NaCl)
Temperature: 310 K
50 ns Production Run
↓
Trajectory Analysis
• RMSD (Global Stability)
• RMSF (Helix-12 Flexibility)
• H-bond Occupancy
• Ligand Stability in Pocket
↓
Binding Free Energy Calculation (MM-PBSA)
↓
Functional Interpretation
Partial Helix-12 Stabilization
↓
Biased GR Conformation
↓
Reduced GRE Overactivation
↓
Preserved NF-κB Suppression
↓
Selective GR Modulation in Thymocytes

• Mechanism of Vamorolone Action

Vamorolone released
↓
High-affinity GR binding
↓
Selective GR modulation
↓

↓ NF-κB transcription (transrepression) | ↓ TNF, IL-6, IL-1β | Reduced inflammation

↓ GRE overactivation (reduced transactivation) | ↓ Bim, pro-apoptotic genes | Prevention of thymocyte apoptosis

• In Vitro Screening

Prodrug Synthesis & QC (HPLC / LC-MS)
↓
Plasma Stability Assay (Human Serum, 0–48 h)
↓
TSSP Cleavage Validation (Selective Release of Vamorolone)
↓
Establish Thymic Co-Culture Model
(Thymic Epithelial Cells + Thymocytes)
↓
Induce Chronic Glucocorticoid Stress (Dexamethasone)
↓
Apply Treatment Groups
(Control | GC | GC+VAM | GC+Prodrug | GC+Tα1)
↓
Functional Assessments
Apoptosis Assay (Annexin V / Caspase-3)
Flow Cytometry (CD4/CD8, TCR-β, CD69)
GRE Reporter (Transactivation)
NF-κB Reporter (Transrepression)
Stromal Markers (Bcl-2, Bim, GR Translocation)
Central Tolerance Markers (AIRE, FoxP3)
Cytokine Profiling (IL-2, IL-7, TNF-α, IL-6)
↓
Data Analysis & Statistical Comparison
↓
Mechanistic Interpretation:

TSSP Cleavage → Local Vamorolone Release → Selective GR Modulation → Reduced Apoptosis → Preserved DP Thymocytes → Maintained Central Tolerance

• In Vivo Validation

Animal Selection
(NOD Female Mice, 6–8 Weeks)
↓
Induce Chronic Glucocorticoid Stress (Dexamethasone OR Restraint Stress)
↓
Randomize Into Treatment Groups (Healthy | GC | GC+VAM | GC+Prodrug | GC+Tα1)
↓
6–12 Week Treatment Period
↓

Primary Thymic Assessments
Thymus Weight (Normalized) Histology (Cortex/Medulla Thickness)
TUNEL Apoptosis Assay
Bcl-2 / Bim Protein Expression
↓

Thymocyte Maturation Analysis
Flow Cytometry:
CD4 / CD8
TCR-β CD69
FoxP3
↓

T-Cell Output Diversity
High-Throughput TCRβ Sequencing
Shannon Diversity Index Calculation
↓

Autoimmunity Assessment
Weekly Blood Glucose Monitoring
Kaplan–Meier Diabetes Incidence
Pancreatic Histology (Insulitis Score)
Autoantibody Levels (Anti-GAD65, Anti-Insulin)
↓

GR Signaling Validation
GRE Target Gene Expression (qPCR)
NF-κB Target Gene Expression
↓

Safety Monitoring
Body Weight
Serum Cortisol
Bone Density Markers
↓

Data Analysis
ANOVA (Multi-group Comparison)
Kaplan–Meier Survival Analysis
TCR Diversity Statistics
↓

Final Outcome Interpretation
Prodrug Cleavage in Thymus
→ Selective GR Modulation
→ Reduced Thymocyte Apoptosis
→ Preserved DP Cells
→ Maintained TCR Diversity
→ Reduced Autoreactive T Cells
→ Delayed Autoimmune Diabetes

2.3 Feasibility

While the mechanism is reasonable, believable, and consistent with available evidence and logic. It must be proven that the SEGRM Vamorolone, once released inside the thymus will have the intended protective effect on stromal cells and thymocyte selection without systemic glucocorticoids receptor modulation. We should

test in-vitro by synthesize the first prototype conjugate and test its stability, cleavage, and thermo-protective effects in cells co-culture models. We are demonstrating prevention of autoimmunity in models like non-obese diabetic mice, it is the gold standard and proving a cause-effect link between thymus protection and autoimmune prevention is necessary for translational impact. We refine the molecule based on results before committing to long term autoimmune disease prevention studies.

These concepts, while speculative, are grounded in known principles:

• Tissue-Selective Action: The success of vamorolone proves that dissociated GR ligands are clinically achievable.

• Thymic Enzyme Targeting: The pro-drug approach mirrors advanced cancer therapies that use tumor-specific enzyme activation.

• Thymosin as a Carrier: Tα1's small size (28 amino acids) and known sequence make it an ideal candidate for peptide engineering and conjugation.

3. Conclusion and Healthcare Implications

Re-engineer Thymosin α-1 Pro drug thus helps thymocytes to build resistance from chronic high glucocorticoids level in body and maintain central tolerance without suppressing normal T cell proliferation. So re-engineered thymosin α-1 pro drug fills the gap of tissue specific glucocorticoid receptor modulator and there is currently no drug available that acts exclusively on the glucocorticoids receptor on thymus to inhibit auto-reactive T cells. By protecting the thymus's ability to enforce central tolerance, this strategy aims to prevent the initial emergence of pathogenic autoreactive T cells and thus prevent auto-immune disorders. This helps to cope-up systemic immune response lead to autoimmune disease such as SLE(Systemic Lupus Erythematosus) involve multiorgan damage due to widespread immune dysregulation, whereas organs specific variants such as Hashimoto's Thyroiditis(HS) and Myasthenia Gravis(MG) predominantly affect specific organs or glands [7].

It directly addresses the side-effects of thymic atrophy and immunosuppression caused by long term glucocorticoid therapy(Example for autoimmune diseases). This could allow for safer, long-term use of this potent anti-inflammatory drugs. By protecting the thymus's ability to enforce central tolerance, this strategy aims to prevent the initial emergence of pathogenic autoreactive T-cells. Unlike broad-spectrum immunosuppressants that weaken the entire immune system, this therapy aims that weaken the entire immune system, this therapy aims to be highly localized to the thymus. In this way, it preserve the patient's ability to fight infection and show effective vaccine responses. Preventing or delaying chronic autoimmune diseases(like Type-1 Diabetes, Rheumatoid Arthritis). This could significantly reduce the life-long costs associated with medication, hospitalizations, management of complication, and lost productivity (working capacity). Success would validate 'Thymus-protective' and 'tolerance-preserving' drugs as a new category in immunology.

Novelty of Design

The core novelty of this design lies in its tissue-specific, dual-action prodrug strategy to pharmacologically enforce central tolerance, a concept not present in existing solutions. Unlike conventional glucocorticoid receptor (GR) modulators or immunosuppressants that act systemically (causing widespread side effects like osteoporosis and general immunosuppression), this design achieves thymus-selective targeting. It uniquely repurposes Thymosin α-1, a natural thymic peptide, as a "homing device" to deliver the SEGRM Vamorolone specifically to the thymic microenvironment. The key differentiator is the engineered protease-sensitive linker. By designing this linker to be cleavable exclusively by Thymus-Specific Serine Protease (TSSP)—an enzyme uniquely present in the thymus—the prodrug remains inert in systemic circulation. Activation occurs only upon reaching the thymus, ensuring that Vamorolone's GR-modulating effects are localized. This prevents the thymic atrophy caused by chronic glucocorticoids while sparing peripheral tissues. Simultaneously, the released Thymosin α-1 exerts its direct anti-apoptotic and differentiation effects on thymocytes. This dual, synchronized release mechanism protects the thymic stromal microenvironment and rescues double-positive thymocytes from glucocorticoid-induced apoptosis, thereby preventing the escape of autoreactive T cells. This represents a shift from broad immunosuppression to targeted immunomodulation aimed at preventing autoimmunity at its developmental origin.

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