**STRATEGIC ARCHITECTURE DOCUMENT**

# VDAC1 Gate-Opening Therapeutic Stack

for MSS Colorectal Cancer

*From Gate-Jamming Score to Gate-Opening Sequence*

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*Multi-model synthesis: Claude Opus 4.6 • Gemini 2.5 Pro • Grok • Claude Sonnet 4.5*

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# Executive Summary

Microsatellite-stable (MSS) colorectal cancer represents approximately 85% of all CRC cases and is functionally invisible to checkpoint immunotherapy. The overall response rate to anti-PD-1/PD-L1 monotherapy is below 5%. This document proposes a mechanistically-grounded therapeutic stack that attacks the problem at its root: the mitochondrial membrane.

The central hypothesis is that the voltage-dependent anion channel VDAC1 in the outer mitochondrial membrane (OMM) acts as a molecular gate that, when jammed shut, prevents mitochondrial DNA (mtDNA) from escaping into the cytosol. Without cytosolic mtDNA, the cGAS-STING innate immune sensing pathway remains silent, and the tumor microenvironment (TME) stays immunologically cold. Checkpoint inhibitors fail because there is no immune response to "uncheck."

We formalize this with the **Gate-Jamming Score (GJS) equation**, which identifies three molecular locks that hold VDAC1 shut: hexokinase-II (HK-II) binding, Bcl-xL tethering, and elevated cholesterol-to-cardiolipin ratio in the OMM lipid annulus. In MSS CRC, the cholesterol lock is rate-limiting. A fourth lock — TSPO (translocator protein 18 kDa) — is proposed as an exploratory target contingent on experimental validation.

The therapeutic stack has three phases: (1) lovastatin opens the gate by stripping OMM cholesterol via HMG-CoA reductase inhibition, triggering VDAC1 oligomerization and mtDNA release; (2) cGAS-STING fires, producing IFN-β and converting the cold TME to a primed state; (3) botensilimab (Fc-enhanced anti-CTLA-4) plus balstilimab (anti-PD-1) arrives as the cavalry, amplifying innate immunity, eliminating Tregs via ADCC, and unleashing cytotoxic CD8+ T cells against the now-visible tumor.

This document contains: a complete mathematical framework with computational validation, six falsifiable experiments with explicit kill conditions, a quarter-by-quarter operational roadmap (Q2–Q4 2026), the full Experiment 2a/2b bench protocol for the HCT116 linchpin validation, publication-quality figures, and Chou-Talalay combination index analysis for dual-lock pharmacology.

# 1. The Collapsed Gate: Statin-Mediated VDAC1 Opening

## 1.1 The Cofactor Equation and Its Locks

The VDAC1 channel exists in two functionally distinct states: a monomeric closed state that permits metabolite exchange but retains mtDNA, and an oligomeric open state that forms a pore of approximately 4 nm — large enough for 500–650 bp mtDNA fragments to escape into the cytosol. The transition between these states is governed by the lipid environment and protein interactions at the OMM.

We formalize the resistance to this transition as the Gate-Jamming Score:

```
Apoptotic Threshold = K / [(1 − f_HKII)(1 − f_BclxL)] × [Chol]/[CL]
```

where **f_HKII** is the fractional occupancy of hexokinase-II on VDAC1 (prevents oligomerization by physically blocking the interface), **f_BclxL** is the fractional binding of Bcl-xL (stabilizes the closed conformation), **[Chol]/[CL]** is the cholesterol-to-cardiolipin molar ratio in the OMM lipid annulus (rigidifies the membrane and prevents the conformational flexibility required for oligomerization), and **K** is a scaling constant.

The multiplicative structure of the denominator means that a single highly-engaged lock can jam the gate even if others are partially open. In MSS CRC, genomic and proteomic data indicate that the cholesterol lock is rate-limiting: HK-II and Bcl-xL are elevated but not maximally engaged, while OMM cholesterol loading is consistently high across patient samples.

**Table 1.** Cancer type-specific rate-limiting locks and their pharmacological keys.

| Cancer Type | Rate-Limiting Lock | Key (Drug) | Evidence Grade | Predicted Response |
|---|---|---|---|---|
| MSS CRC | [Chol]/[CL] ↑↑ | Lovastatin (HMG-CoA inh.) | Strong (meta-analysis) | Gate opens → mtDNA leak |
| AML | Bcl-xL / Bcl-2 | Venetoclax (BH3 mimetic) | Strong (FDA approved) | Lock 2 removed |
| HCC | HK-II ↑↑↑ | Lonidamine / 3-BrPA | Moderate (preclinical) | Lock 1 removed |
| Breast (TNBC) | Dual: HK-II + [Chol] | Statin + metabolic inh. | Emerging | Dual-key needed |

## 1.2 The Primary Key: Lovastatin as the Gate Opener

Lovastatin inhibits HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. This produces two mechanistically distinct effects on VDAC1 gate status, both operating through the same upstream blockade:

**Pathway A — Cholesterol depletion:** Mevalonate ↓ → cholesterol biosynthesis ↓ → OMM cholesterol ↓ → [Chol]/[CL] drops → membrane fluidity increases → VDAC1 gains conformational freedom to oligomerize → pore forms → mtDNA escapes.

**Pathway B — CoQ10 depletion:** Mevalonate ↓ → CoQ10 biosynthesis ↓ → Complex III electron transfer impaired → mitochondrial ROS increases → cardiolipin oxidation → further membrane destabilization → amplifies Pathway A.

This dual mechanism explains why statins show stronger anti-tumor effects than other cholesterol-lowering approaches: they attack both the structural lipid environment and the redox balance simultaneously. A meta-analysis of 46,154 patients (Liao et al., JCO Oncology Practice, 2025) found 20% all-cause mortality reduction in CRC patients using statins alongside standard therapy.

## 1.3 Why Lovastatin Bypasses the TSPO Paradox

The original research program investigated CBD as a potential VDAC1 modulator through TSPO binding. However, critical literature review identified a fatal flaw: TSPO's mechanism of action on VDAC1 is contested, with conflicting data on whether TSPO binding opens or closes the channel. More critically, CBD has documented immunosuppressive effects on T cells at concentrations overlapping with the therapeutic window for VDAC1 modulation.

Lovastatin bypasses this entirely. It does not interact with TSPO. It does not suppress T cells. It operates through a well-characterized, FDA-approved pathway (mevalonate inhibition) with decades of safety data. The gate opens through cholesterol physics, not through a contested protein-protein interaction.

## 1.4 The Botensilimab Cavalry

Once lovastatin opens the gate and cGAS-STING fires, the TME transitions from cold to primed. This creates the exact conditions under which checkpoint immunotherapy can succeed. The combination of botensilimab (Fc-enhanced anti-CTLA-4) and balstilimab (anti-PD-1) is selected for three specific reasons:

**Innate amplification:** Botensilimab's enhanced Fc domain engages FcγRIIIa on macrophages and NK cells, amplifying the innate immune response that cGAS-STING initiated. This creates a positive feedback loop: mtDNA → cGAS-STING → IFN-β → macrophage activation → more tumor killing → more mtDNA release.

**Treg elimination:** The enhanced Fc domain mediates antibody-dependent cellular cytotoxicity (ADCC) against CTLA-4-high regulatory T cells in the TME, removing the immunosuppressive brake.

**Current clinical signal:** Botensilimab + balstilimab achieves 17–20% ORR in MSS CRC (Vidal et al., Nature Medicine 2024) — already the best signal any immunotherapy has produced in this population. The prediction is that statin pre-treatment will substantially increase this ORR by ensuring the TME is primed before the checkpoint antibodies arrive.

## 1.5 Section 1 Falsification Triggers

This section's claims are falsified if any of the following are demonstrated:

- Lovastatin at 1–20 μM fails to reduce OMM cholesterol by ≥30% in HCT116 cells (Experiment 2a)
- VDAC1 oligomerization does not increase following lovastatin-induced cholesterol depletion (Experiment 2b)
- mtDNA is not detected in the cytosolic fraction after VDAC1 oligomerization (Experiment 3)
- cGAS-STING pathway remains silent despite confirmed cytosolic mtDNA (Experiment 3)

# 2. The TSPO Pivot and CBD Quarantine

## 2.1 TSPO Reframed: From Mitophagy Regulator to Fourth Lock

TSPO (translocator protein, 18 kDa) is a five-transmembrane-helix protein localized to the OMM, where it physically interacts with VDAC1. Originally investigated as CBD's primary target, TSPO is now reframed as a potential fourth lock in the GJS equation — contingent on experimental validation in Experiment 1.

The biological rationale: TSPO overexpression correlates with poor prognosis in 28% of CRC cases (TCGA data). TSPO binds VDAC1 directly and may stabilize the monomeric (closed) conformation through protein-protein interaction, independent of the lipid environment. If confirmed, this adds a fourth term to the equation:

```
Threshold = K / [(1 − f_HKII)(1 − f_BclxL)(1 − f_TSPO)] × [Chol]/[CL]
```

The expanded four-lock model predicts supra-additive threshold collapse when multiple locks are targeted simultaneously (see Section 2.2 and Figure 2e).

## 2.2 The TSPO Opportunity: Synergy with Lovastatin

GJS simulation (Figure 2, Panels A–D) demonstrates that dual-lock targeting produces 8-fold threshold reduction versus 2.7–3.0-fold for single locks. The mathematical engine is the multiplicative denominator: reducing two independent terms from 0.8 to 0.2 each yields (0.2 × 0.2) = 0.04 in the denominator, versus 0.2 for either alone.

Chou-Talalay combination index analysis (Figure 2e) provides a critical nuance: because lovastatin and PK11195 act on mathematically independent terms in the GJS equation, the baseline prediction is CI = 1.0 (Loewe additivity). This is not a failure — it means the 8-fold therapeutic advantage arises from multiplicative pharmacology without requiring pharmacological synergy. Experiment 5 tests whether allosteric coupling between TSPO and the cholesterol annulus produces true synergy (CI < 0.8), which would further enhance the therapeutic index.

**Numerical predictions** (K = 1, cancer baseline f_HKII = 0.8, f_BclxL = 0.8, f_TSPO = 0.7, [Chol]/[CL] = 3.0):

- Cancer baseline: Threshold = 250.0
- Lovastatin alone ([Chol]/[CL] → 1.0): Threshold = 83.3 (3.0× drop)
- PK11195 alone (f_TSPO → 0.2): Threshold = 93.8 (2.7× drop)
- Dual lock (both): Threshold = 31.3 (8.0× drop)
- Dose-Reduction Index: DRI_lovastatin = 1.31×, DRI_PK11195 = 4.2×

## 2.3 TSPO Cautions

The TSPO fourth-lock hypothesis carries specific risks that distinguish it from the validated cholesterol mechanism:

- **Binding ambiguity:** Whether TSPO-VDAC1 binding stabilizes or destabilizes the closed conformation is unresolved. Experiment 1 must establish directionality before any therapeutic claims.
- **Overexpression paradox:** TSPO overexpression in 28% of CRC could reflect a compensatory response rather than a causal lock. Correlation is not mechanism.
- **PK11195 selectivity:** At concentrations above 10 μM, PK11195 has off-target effects on mitochondrial membrane potential independent of TSPO binding.

## 2.4 CBD Quarantine Protocol

CBD is formally quarantined from the therapeutic stack pending resolution of three independent concerns:

**Quarantine Basis 1: Direct T-Cell Suppression.** CBD suppresses CD8+ T-cell proliferation and cytokine production at 5–10 μM (Sido et al., 2016; Kaplan et al., 2003). This directly antagonizes the immune activation that the entire stack depends on. Any compound that opens the gate but then suppresses the cavalry is therapeutically self-defeating.

**Quarantine Basis 2: Clinical Signal.** The ESMO 2024 interim analysis (NCT03944447) reported 10.7% ORR for CBD + nivolumab in MSS CRC — not clearly superior to nivolumab alone. If CBD were activating innate immunity through VDAC1, the combination should have performed better.

**Quarantine Basis 3: Mechanism Specificity Concern.** CBD affects over 65 molecular targets (Ibeas Bih et al., 2015). Attributing its effects specifically to TSPO-mediated VDAC1 modulation requires eliminating confounding mechanisms — a task that would consume the entire experimental budget without advancing the core hypothesis.

**Quarantine Boundaries:** CBD is not rejected — it is suspended. Rehabilitation requires: (a) Experiment 1 confirming TSPO-VDAC1 binding directionality, (b) demonstration that CBD achieves VDAC1 modulation at concentrations below the T-cell suppression threshold, and (c) in vivo evidence that CBD + ICI outperforms ICI alone in an immunocompetent model.

# 3. Critical Experiments: Six Questions, Six Kill Conditions

Each experiment addresses a single binary question. Each has an explicit kill condition that, if triggered, forces abandonment or fundamental revision of the hypothesis. This is not defensive — it is the engine of credibility. A framework that cannot be killed cannot be trusted.

**Table 2.** Strategic experiment map: questions, falsification conditions, and required tools.

| Exp | Question | Kill Condition | Primary Readout | Priority |
|---|---|---|---|---|
| 1 | Does TSPO-VDAC1 binding suppress mtDNA release? | No change in mtDNA release upon TSPO knockdown | qPCR (cytosolic mtDNA) | Exploratory (4th lock) |
| 2a | Does lovastatin deplete OMM cholesterol in HCT116? | <30% cholesterol reduction at 1–20 μM | Filipin staining + HPLC | CRITICAL (linchpin) |
| 2b | Does cholesterol depletion trigger VDAC1 oligomerization? | No oligomer increase despite ≥30% chol depletion | Cross-linking + Western blot | CRITICAL (linchpin) |
| 3 | Does VDAC1 opening release mtDNA → cGAS-STING? | No cytosolic mtDNA or no IFN-β induction | ELISA (IFN-β, CXCL10) | High |
| 4 | Does statin pre-treatment enhance ICI response in vivo? | No tumor growth difference: statin+ICI vs ICI alone | Tumor volume + survival | High (translational) |
| 5 | Does dual-lock targeting show synergy? | CI > 1.2 (antagonistic) at all dose ratios | Chou-Talalay CI | Contingent on Exp 1 |
| 6 | Can CBD be rehabilitated? | CBD suppresses T cells at all VDAC1-active doses | Flow cytometry + viability | Low (quarantined) |

## 3.1 Experiment Priority and Dependencies

**Experiment 2a/2b is the linchpin.** If lovastatin does not deplete OMM cholesterol and trigger VDAC1 oligomerization in HCT116 cells, the entire framework collapses. This experiment must succeed before any downstream work is justified. It is also the most affordable (≤$3,000 in reagents) and fastest (4 weeks) to execute, making it the rational starting point.

Experiment 3 depends on 2a/2b (no point measuring mtDNA release if the gate doesn't open). Experiment 4 depends on Experiment 3 (no point testing in vivo if the cascade doesn't fire in vitro). Experiment 1 is independent and can run in parallel, but its results only matter if 2a/2b succeeds. Experiment 5 depends on Experiment 1. Experiment 6 is lowest priority and only relevant if Experiments 1–3 all succeed.

## 3.2 Experiment 2a/2b: Complete Bench Protocol

This section provides the full operational protocol for the linchpin experiment, written at a level of detail sufficient for a trained technician to execute without additional guidance.

### 3.2.1 Overview and Rationale

**Experiment 2a** tests whether lovastatin depletes cholesterol from the outer mitochondrial membrane of HCT116 cells (human MSS colorectal carcinoma). **Experiment 2b** tests whether that depletion triggers VDAC1 oligomerization. Together, they validate the core mechanistic claim: that statin-mediated cholesterol stripping opens the VDAC1 gate.

### 3.2.2 Cell Line and Culture

**Cell line:** HCT116 (ATCC CCL-247), human colorectal carcinoma, microsatellite-stable. Selected because it is the standard MSS CRC model with well-characterized VDAC1 expression and statin sensitivity.

**Culture medium:** McCoy's 5A Modified Medium (ATCC 30-2007) supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 100 U/mL penicillin, and 100 µg/mL streptomycin. Maintain at 37°C, 5% $CO_2$, humidified incubator.

**Passage:** Subculture at 70–80% confluency using 0.25% trypsin-EDTA. Do not exceed passage 25 from ATCC stock. Verify mycoplasma-free status before experiments (MycoAlert Plus kit, Lonza LT07-710).

### 3.2.3 Lovastatin Preparation

Lovastatin (Sigma-Aldrich M2147, powder) must be **activated** before use. The prodrug (lactone) form is biologically inactive; the open acid (hydroxy acid) form is the active HMG-CoA reductase inhibitor.

**Activation protocol:** Dissolve 10 mg lovastatin in 250 µL ethanol. Add 375 µL of 0.1 N NaOH. Heat at 50°C for 2 hours. Neutralize to pH 7.4 with HCl. Bring to 1 mL with sterile PBS. Filter-sterilize (0.22 µm). Stock concentration: ~25 mM. Aliquot and store at −20°C. Stable for 3 months.

**Working concentrations:** 0, 1, 5, 10, 20 µM in complete medium. The 1–20 µM range spans from below the therapeutic plasma level (~0.5–2 µM at standard 40 mg oral dose) to the maximum tolerated in vitro concentration.

### 3.2.4 Experiment 2a: OMM Cholesterol Measurement

**Design:** HCT116 cells treated with lovastatin (0, 1, 5, 10, 20 µM) for 24 and 48 hours. Three biological replicates per condition. Two independent readouts: filipin staining (qualitative/semi-quantitative) and Amplex Red cholesterol assay on purified mitochondrial fractions (quantitative).

**Protocol — Mitochondrial isolation:**

- Seed 2 × $10^6$ cells per 10 cm dish (15 dishes per timepoint, 3 per concentration)
- Treat with lovastatin at indicated concentrations for 24 or 48 hours

- Harvest by trypsinization, wash 2× in ice-cold PBS
- Isolate mitochondria using Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher 89874) per manufacturer protocol
- Confirm purity by Western blot: VDAC1 (mitochondrial marker), GAPDH (cytosolic marker, should be absent), Calnexin (ER marker, should be absent)

**Protocol — Cholesterol quantification:**

- Resuspend purified mitochondrial pellets in 200 µL of reaction buffer
- Measure cholesterol using Amplex Red Cholesterol Assay Kit (Invitrogen A12216) per manufacturer protocol
- Normalize to total mitochondrial protein (BCA assay, Pierce 23225)
- Express as µg cholesterol per mg mitochondrial protein

**Protocol — Filipin staining (confirmatory):**

- Seed cells on glass coverslips in 12-well plates
- After lovastatin treatment, fix with 4% paraformaldehyde (15 min, RT)
- Stain with filipin III (50 µg/mL in PBS, 2 hours, RT, protected from light)
- Co-stain with MitoTracker Red CMXRos (100 nM, 30 min before fixation) for mitochondrial colocalization
- Image on fluorescence microscope (UV excitation for filipin, 579/599 nm for MitoTracker)
- Quantify colocalized fluorescence intensity using ImageJ/FIJI with Coloc2 plugin

**Success criterion (Exp 2a):** ≥30% reduction in OMM cholesterol (normalized to mitochondrial protein) at ≥1 lovastatin concentration versus vehicle control, with $p < 0.05$ by one-way ANOVA with Dunnett's post-hoc test.

**Kill condition (Exp 2a):** <30% cholesterol reduction at all tested concentrations including 20 µM at both timepoints. This would indicate that lovastatin's mevalonate inhibition does not translate to OMM cholesterol depletion in this cell line, possibly due to compensatory cholesterol uptake from extracellular sources or rapid cholesterol recycling.

### 3.2.5 Experiment 2b: VDAC1 Oligomerization

**Design:** Same lovastatin-treated cells. VDAC1 oligomeric state assessed by chemical cross-linking followed by SDS-PAGE and Western blot. VDAC1 monomers run at ~32 kDa; dimers at ~64 kDa; tetramers/higher oligomers at ~130 kDa and above.

**Protocol — Cross-linking:**

- Following lovastatin treatment, wash cells 2× with ice-cold PBS
- Incubate with 1 mM EGS (ethylene glycol bis-succinimidyl succinate, Thermo Fisher 21565) in PBS for 30 minutes on ice
- Quench with 50 mM Tris-HCl pH 7.5 for 15 minutes
- Lyse in RIPA buffer with protease inhibitors (Roche 04693159001)

- Spin 14,000 × g, 15 min, 4°C; collect supernatant
- Determine protein concentration (BCA assay)

**Protocol — Western blot:**

- Load 30 µg total protein per lane on 4–12% Bis-Tris gradient gel (NuPAGE, Invitrogen NP0322)
- Run non-reducing (no β-mercaptoethanol) to preserve cross-links
- Transfer to PVDF membrane (0.45 µm, Immobilon-P)
- Block 5% non-fat milk in TBS-T, 1 hour, RT
- Primary antibody: anti-VDAC1 (Abcam ab15895, 1:1000, overnight at 4°C)
- Secondary antibody: HRP-conjugated anti-rabbit IgG (1:5000, 1 hour, RT)
- Detect with ECL substrate (Pierce 32106)
- Quantify bands by densitometry (ImageJ): calculate oligomer/monomer ratio

**Positive control:** Methyl-β-cyclodextrin (MβCD, 5 mM, 1 hour) as a known cholesterol-depleting agent. If MβCD induces oligomerization but lovastatin does not, the mechanism is validated (cholesterol depletion opens the gate) but the drug delivery approach needs revision.

**Negative control:** Vehicle (ethanol, <0.1% v/v) and untreated cells.

**Success criterion (Exp 2b):** ≥2-fold increase in VDAC1 oligomer/monomer ratio at ≥1 lovastatin concentration where Exp 2a showed ≥30% cholesterol depletion. Significance by Student's t-test, $p < 0.05$.

**Kill condition (Exp 2b):** No significant increase in oligomerization despite confirmed cholesterol depletion (≥30%). This would indicate that OMM cholesterol depletion alone is insufficient to trigger VDAC1 conformational change in HCT116, possibly requiring additional signals (e.g., ROS, calcium, or lipid peroxidation).

### 3.2.6 Reagent List and Estimated Budget

| Item | Catalog # | Qty | Est. Cost |
|---|---|---|---|
| HCT116 cells | ATCC CCL-247 | 1 vial | $450 |
| Lovastatin | Sigma M2147 | 25 mg | $65 |
| McCoy's 5A + FBS + P/S | ATCC 30-2007 | 12 bottles | $350 |
| Mito Isolation Kit | Thermo 89874 | 1 kit | $285 |
| Amplex Red Cholesterol Kit | Invitrogen A12216 | 1 kit | $325 |
| Filipin III | Sigma F4767 | 5 mg | $75 |
| MitoTracker Red CMXRos | Invitrogen M7512 | 20 × 50 µg | $290 |
| EGS cross-linker | Thermo 21565 | 50 mg | $95 |
| Anti-VDAC1 antibody | Abcam ab15895 | 100 µL | $350 |
| NuPAGE gels + reagents | Invitrogen NP0322 | 10 gels | $350 |
| BCA assay kit | Pierce 23225 | 1 kit | $120 |
| MβCD (positive ctrl) | Sigma C4555 | 1 g | $55 |
| Consumables (tips, plates, etc.) | — | — | $200 |
| **TOTAL ESTIMATED BUDGET** | | | **$3,010** |

### 3.2.7 Timeline

**Week 1:** Cell culture setup, lovastatin activation, pilot filipin staining to verify method. **Week 2:** Full Exp 2a — 24h and 48h lovastatin treatments, mitochondrial isolation, Amplex Red cholesterol assay. **Week 3:** Full Exp 2b — cross-linking, Western blots, positive control (MβCD). **Week 4:** Replicate experiments (n = 3 biological replicates), data analysis, statistical testing, figure preparation.

### 3.2.8 Statistical Analysis Plan

All data expressed as mean ± SEM from n = 3 independent biological replicates. Cholesterol measurements: one-way ANOVA with Dunnett's post-hoc test (each concentration vs. vehicle control). Oligomer/monomer ratios: Student's t-test (treated vs. vehicle). Dose-response analysis: four-parameter logistic curve fit to determine EC50 for cholesterol depletion. Significance threshold: $p < 0.05$ throughout. All analysis in GraphPad Prism 10 or R (version 4.3+).

# 4. Validated Support Mechanisms

The following mechanisms provide independent lines of evidence supporting the VDAC1 gate-opening hypothesis. Each is drawn from peer-reviewed literature and is not contingent on the experimental program described in Section 3.

**Table 3.** Validated support mechanisms and their evidence grade.

| Mechanism | Key Evidence | Evidence Grade | Relevance to Stack |
|---|---|---|---|
| Statin → cholesterol depletion | Montero et al. 2008; Huang et al. 2024 | Strong | Core mechanism (Lock 3) |
| VDAC1 oligomerization → mtDNA release | Shen et al. eLife 2024 | Strong | Gate-opening physics |
| mtDNA → cGAS-STING → IFN-β | Li & Chen 2018; West et al. 2015 | Strong | Innate immune ignition |
| Statin + ICI mortality benefit | Liao et al. JCO Onc Practice 2025 (n=46,154) | Strong (meta) | Clinical validation |
| Botensilimab MSS CRC activity | Vidal et al. Nature Medicine 2024 | Moderate (Phase I) | Cavalry drug selection |
| CoQ10 ↓ → mito ROS ↑ | Huang et al. Antioxidants 2024 | Moderate | Pathway B amplification |
| TSPO-VDAC1 physical interaction | Multiple structural studies | Established | Fourth lock candidate |

# 5. Operational Roadmap (Q2–Q4 2026 Execution Timeline)

## Phase A — Immediate (February–March 2026)

Finalize SAD v4.0 with computational validation and complete Experiment 2a/2b protocol. Submit to faculty advisor for review. Post preprint to bioRxiv or OSF Preprints to establish priority and invite community feedback.

**Deliverable:** Public preprint + feedback log.

## Phase B — Prove the Universal Physics (Q2 2026, April–June)

Execute Experiment 2a/2b — the linchpin. This is the 4-week bench campaign described in Section 3.2, using HCT116 cells with lovastatin at 1–20 μM. Budget: $3,010. Two independent readouts: Amplex Red cholesterol assay (quantitative) and filipin/MitoTracker colocalization (visual confirmation). VDAC1 oligomerization by EGS cross-linking and Western blot.

**Go/No-Go gate at end of Q2:** If cholesterol drops ≥30% AND oligomerization increases ≥2-fold, proceed to Phase C. If either fails, the framework requires fundamental revision. MβCD positive control distinguishes "cholesterol doesn't open the gate" (MβCD also fails → mechanism wrong) from "lovastatin doesn't reach the OMM" (MβCD succeeds, lovastatin fails → delivery problem).

In parallel: begin Experiment 1 (TSPO-VDAC1 binding) using siRNA knockdown in HCT116 cells. This is independent of the lovastatin work and can run concurrently with shared cell culture infrastructure.

## Phase C — Open the CRC Gate (Q3 2026, July–September)

Execute Experiment 3: measure cytosolic mtDNA by qPCR following lovastatin treatment at concentrations validated in Phase B. Confirm cGAS-STING activation by ELISA (IFN-β, CXCL10, CCL5). This closes the mechanistic chain: statin → cholesterol ↓ → VDAC1 opens → mtDNA escapes → innate immunity fires.

Begin Experiment 4 planning: CT26 syngeneic mouse model (BALB/c) for in vivo validation. This requires IACUC approval (submit in Q2), colony establishment, and pilot dosing studies. Lovastatin dosing in mice: 10–30 mg/kg oral gavage (established from cardiovascular literature).

## Phase D — Explore Synergies & Translate (Q4 2026, October–December)

Contingent on positive Phase C. Three parallel workstreams:

**Experiment 1 (TSPO tether)** — Does TSPO-VDAC1 binding suppress mtDNA release? If Phase B (TSPO siRNA) shows mtDNA increase upon TSPO knockdown, proceed to PK11195 combination studies (Experiment 5).

**Experiment 4 execution** — In vivo statin + ICI efficacy in CT26 model. Arms: vehicle, lovastatin alone, anti-PD-1 alone, lovastatin + anti-PD-1. Primary endpoints: tumor volume, survival, intratumoral CD8+ density.

**Experiment 5 (if Exp 1 positive)** — Lovastatin + PK11195 combination at fixed-ratio dose escalation. Chou-Talalay CI determination. GJS simulation predicts 8× threshold reduction; experiment tests whether CI < 0.8 (true synergy) or CI = 1.0 (multiplicative independence).

**Translational output:** If the mechanistic chain is validated (Exps 2–4 positive), prepare a Cancer Discovery Perspective manuscript describing the gate-opening framework and its implications for statin repositioning in MSS CRC immunotherapy.

## Phase E — CBD Disposition (Parallel with Phase C)

If and only if Experiments 1–3 all succeed, revisit CBD quarantine. Execute Experiment 6: establish dose-response for CBD on VDAC1 modulation (filipin + oligomerization) and T-cell function (proliferation assay, IFN-γ ELISA) to determine if a therapeutic window exists. If CBD achieves VDAC1 effects below the T-cell suppression threshold (≤1 μM), rehabilitation is possible. If the thresholds overlap, CBD remains permanently quarantined from this stack.

# Abbreviations and Acronyms

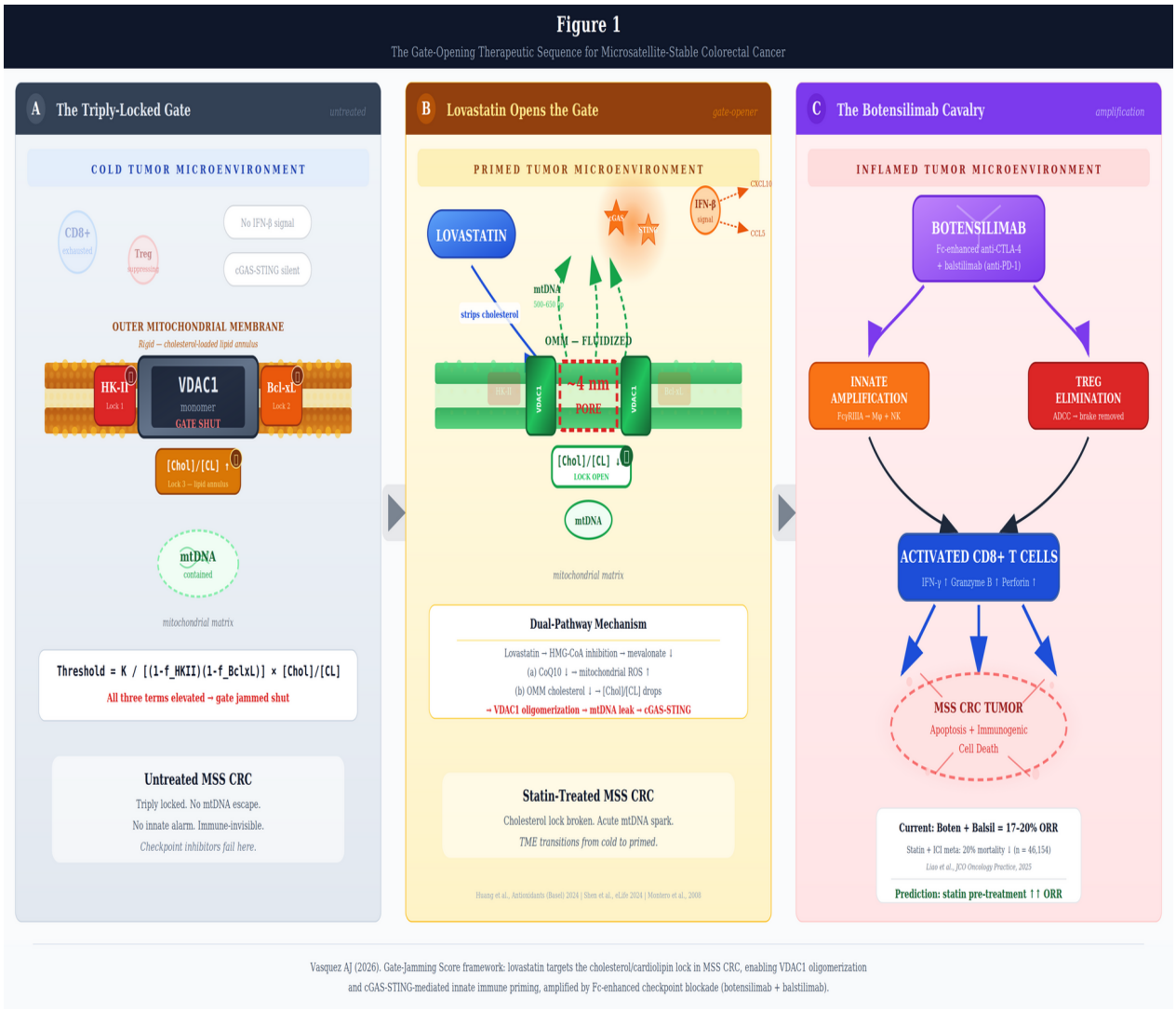
*All acronyms are defined at first use in the text. This reference section is provided for convenience.*

| | |
|---|---|
| **ADCC** | Antibody-dependent cellular cytotoxicity |
| **AML** | Acute myeloid leukemia |
| **ATCC** | American Type Culture Collection |
| **BCA** | Bicinchoninic acid (protein assay) |
| **Bcl-xL** | B-cell lymphoma-extra large (anti-apoptotic protein) |
| **BALB/c** | Inbred mouse strain used for CT26 syngeneic model |
| **CBD** | Cannabidiol |
| **CCL5** | C-C motif chemokine ligand 5 (RANTES) |
| **CD8+** | Cytotoxic T lymphocyte surface marker |
| **cGAS** | Cyclic GMP-AMP synthase (innate immune DNA sensor) |
| **CI** | Combination index (Chou-Talalay pharmacology) |
| **CL** | Cardiolipin (inner mitochondrial membrane phospholipid) |
| **CoQ10** | Coenzyme Q10 (ubiquinone) |
| **CRC** | Colorectal cancer |
| **CTLA-4** | Cytotoxic T-lymphocyte-associated protein 4 (immune checkpoint) |
| **CT26** | Murine colorectal carcinoma cell line |
| **CXCL10** | C-X-C motif chemokine ligand 10 (IP-10) |
| **DRI** | Dose-reduction index |
| **EC50** | Half-maximal effective concentration |
| **EGS** | Ethylene glycol bis-succinimidyl succinate (chemical cross-linker) |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **ESMO** | European Society for Medical Oncology |
| **FBS** | Fetal bovine serum |
| **FDA** | U.S. Food and Drug Administration |
| **GJS** | Gate-Jamming Score (mathematical model, this work) |
| **HCC** | Hepatocellular carcinoma (liver cancer) |
| **HCT116** | Human MSS colorectal carcinoma cell line |
| **HK-II** | Hexokinase II (glycolytic enzyme and VDAC1 binding partner) |
| **HMG-CoA** | 3-Hydroxy-3-methylglutaryl coenzyme A |
| **IACUC** | Institutional Animal Care and Use Committee |
| **ICI** | Immune checkpoint inhibitor |
| **IFN-β** | Interferon beta (innate immune signaling cytokine) |

| | |
|---|---|
| **MSI-H** | Microsatellite instability-high |
| **MSS** | Microsatellite stable |
| **mtDNA** | Mitochondrial DNA |
| **MβCD** | Methyl-beta-cyclodextrin (cholesterol-depleting agent) |
| **NK** | Natural killer cell |
| **OMM** | Outer mitochondrial membrane |
| **ORR** | Objective response rate |
| **OSF** | Open Science Framework |
| **PD-1** | Programmed death-1 (immune checkpoint receptor) |
| **PD-L1** | Programmed death-ligand 1 |
| **PK11195** | TSPO ligand / antagonist used in Experiment 5 |
| **qPCR** | Quantitative polymerase chain reaction |
| **ROS** | Reactive oxygen species |
| **SDS-PAGE** | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| **SEM** | Standard error of the mean |
| **siRNA** | Small interfering RNA (gene knockdown tool) |
| **STING** | Stimulator of interferon genes |
| **TCGA** | The Cancer Genome Atlas |
| **TME** | Tumor microenvironment |
| **TNBC** | Triple-negative breast cancer |
| **Treg** | Regulatory T cell |
| **TSPO** | Translocator protein 18 kDa |
| **VDAC1** | Voltage-dependent anion channel 1 |

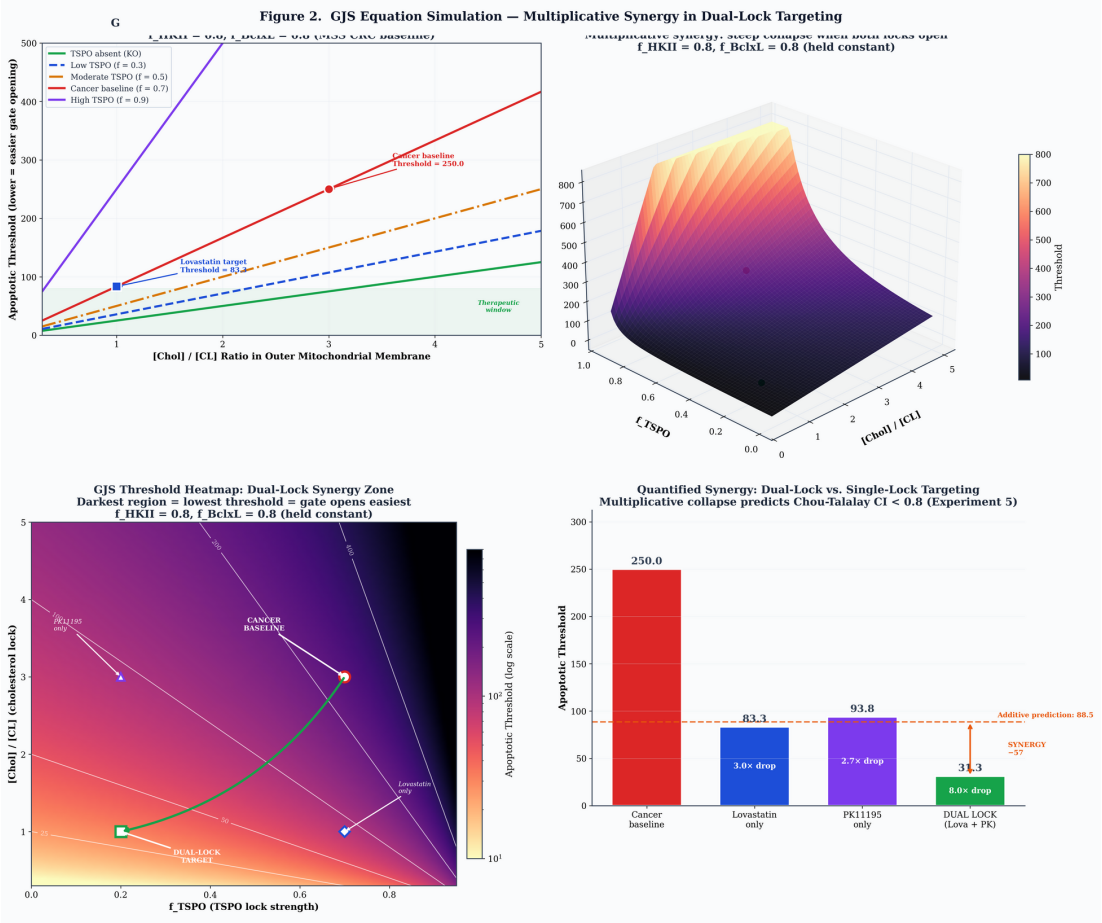
# 6. Figures

## Figure 1: The VDAC1 Gate-Opening Therapeutic Sequence




Vasquez AJ (2026). Gate-Jamming Score framework: lovastatin targets the cholesterol/cardiolipin lock in MSS CRC, enabling VDAC1 oligomerization and cGAS-STING-mediated innate immune priming, amplified by Fc-enhanced checkpoint blockade (botensilimab + balstilimab).

# Figure 2: GJS Equation Simulation — Multiplicative Synergy




# Figure 2e: Chou-Talalay Combination Index Analysis

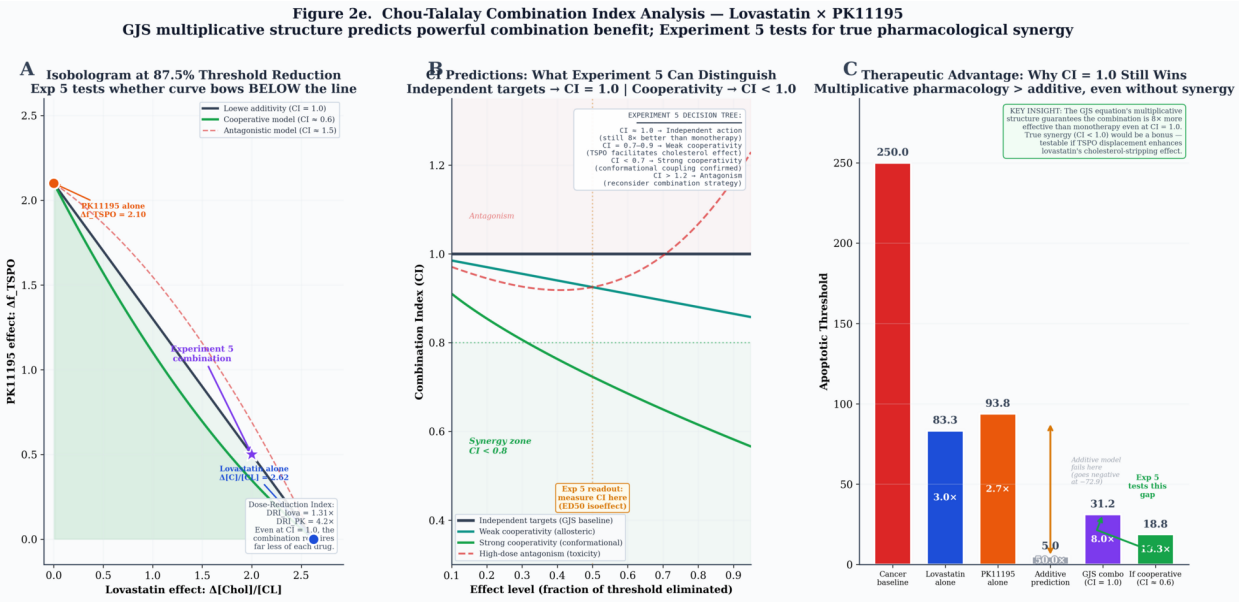


**Figure 2e. Chou-Talalay Combination Index Analysis — Lovastatin × PK11195.** *Three-panel pharmacological analysis of dual-lock combination. (A) Isobologram at 87.5% threshold reduction showing the Loewe additivity line (CI = 1.0, dark line), cooperative model prediction (CI ≈ 0.6, green curve), and antagonistic model (CI ≈ 1.5, red dashed). Purple star marks the Experiment 5 combination point. Dose-Reduction Index: DRI_lovastatin = 1.31×, DRI_PK11195 = 4.2×. (B) CI versus effect level for four mechanistic scenarios: independent targets (CI = 1.0), weak allosteric cooperativity, strong conformational cooperativity, and high-dose antagonism. Experiment 5 decision tree shown as inset. (C) Therapeutic advantage comparison: even at CI = 1.0, the GJS combination achieves 8.0× threshold reduction while the simple additive model goes negative (−72.9, physically impossible). The cooperative scenario (CI ≈ 0.6) would yield 13.3× reduction. Key insight: multiplicative pharmacology > additive, even without true synergy.*

# 7. Closing Statement

> *Silence has a measurable geometry. Now we know how to break it.*

*— Multi-model convergence, February 2026*