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Article

From Undruggable to Therapeutic: Housekeeping Gene Expression Patterns in Human Peripheral Blood Mononuclear Cells Treated with Metadichol

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Abstract

Background: Housekeeping genes (HKGs) are fundamental cellular components essential for maintaining basic cellular functions including metabolism, protein synthesis, and structural integrity. For decades, these genes were considered "undruggable" due to their fundamental role in cell survival. However, recent advances in cancer biology have revealed that cancer cells exhibit altered dependence on housekeeping genes—particularly those involved in glycolysis (Warburg effect) and protein synthesis—creating therapeutic opportunities. Analysis of single-cell RNA sequencing data from the Human Protein Atlas reveals that these genes demonstrate ubiquitous expression across 557 human cell type clusters, confirming their essential roles in cellular homeostasis. Metadichol, a nanoemulsion of policosanols, functions as a 'master conductor' of gene regulation, modulating all 49 nuclear receptors, sirtuins (SIRT1-7), Yamanaka factors, tumor suppressors, and numerous other master regulatory pathways. Unlike direct enzyme inhibitors that carry significant toxicity risks, Metadichol achieves bidirectional gene regulation at picomolar and nanomolar concentrations with a highly favorable safety profile (LD50 >5000 mg/kg). **Methods:** PBMCs were isolated from fresh human blood using density gradient centrifugation and treated with Metadichol at concentrations ranging from 0.1 pg/mL to 100 ng/mL for 24 hours. Gene expression analysis was performed using quantitative real-time PCR (qRT-PCR) with SYBR Green detection chemistry. Nineteen housekeeping genes spanning six functional categories were analyzed: cytoskeletal proteins (ACTB, TUBB), translation factors (EIF1, EIF2S2, EIF4A2, EIF5, RPLP0), heat shock proteins (HSPA5, HSPA9, CANX), stress response transcription factors (ATF4, HIF1A), signaling molecules (PTPRO, PTMS, PTMA, B2M), and metabolic enzymes (GAPDH, HPRT1, FDFT1). Expression data were normalized to GAPDH using the $2^{-\Delta\Delta C_q}$ method. Correlation analysis and gene network mapping were performed to identify co-expression patterns. **Results:** Significant modulation was observed at concentrations as low as 0.1 pg/mL (10^{-13} g/mL). The stress response transcription factor ATF4 showed robust upregulation (4.06-fold at 1 pg/mL, $P < 0.05$), representing activation of the integrated stress response pathway. The tumor suppressor PTPRO demonstrated the highest fold induction observed (6.02-fold at 100 pg/mL, $P < 0.001$), with a unique triphasic dose-response pattern. HIF1A exhibited biphasic regulation (1.61× at 0.1 pg/mL, 0.45× at 1 pg/mL, $P < 0.05$), reflecting mTOR/SIRT1 pathway interplay. Classical housekeeping genes ACTB (0.94-1.09×) and TUBB (0.86-1.12×) maintained stable expression, validating selective targeting. Heat shock proteins HSPA5, HSPA9, and CANX showed concentration-specific modulation supporting enhanced proteostasis. Gene-gene correlation analysis revealed coordinated regulatory modules. **Conclusions:** This study demonstrates that Metadichol selectively modulates stress-responsive housekeeping genes while preserving core cellular machinery. We propose that housekeeping gene modulation represents the unifying upstream mechanism explaining Metadichol's diverse biological effects through hierarchical network amplification. The 19 housekeeping genes include master regulators (ATF4, HIF1A, EIF factors) that each control hundreds of downstream targets, explaining how picomolar concentrations produce broad therapeutic effects. This positions Metadichol as a first-in-class compound capable of comprehensive housekeeping gene modulation through a physiological nuclear receptor-mediated mechanism.

Keywords: housekeeping genes; metadichol; policosanol; gene expression; PBMCs; ATF4; PTPRO; integrated stress response; nuclear receptors; vitamin D receptor; qRT-PCR; dose-response; gene regulatory networks; mTOR; cellular reprogramming; ultra-low dose therapeutics

Introduction

Historical Perspective: Why Housekeeping Genes Were Avoided

Housekeeping genes (HKGs) constitute a fundamental category of genes expressed constitutively in all cell types to maintain essential cellular functions necessary for cell survival and basic cellular processes.[1–3] These genes encode proteins involved in critical biological pathways including glycolysis, protein synthesis, cytoskeletal organization, cellular metabolism, and stress response mechanisms. The defining characteristic of HKGs is their relatively stable expression across various tissues, developmental stages, and experimental conditions.

For decades, the pharmaceutical industry avoided targeting these genes due to concerns about systemic toxicity arising from their ubiquitous expression.[4,5] The prevailing dogma held that inhibiting genes essential for basic cellular survival would inevitably harm normal cells alongside diseased ones, creating an unacceptable therapeutic index. This view was reinforced by several compelling observations:

Essential for Cell Survival: HKGs encode proteins required for cytoskeletal integrity, protein synthesis, and energy metabolism. Complete inhibition would be lethal to all cells.[5,6]

Ubiquitous Expression: Expression in all cell types raised toxicity concerns. Targeting would affect healthy and diseased cells equally.[7,8]

Evolutionary Conservation: HKGs are highly conserved across species. Mutations are often embryonic lethal.[9,10]

Stable Expression by Design: The very characteristic that makes HKGs useful as reference genes—their stable expression—makes them poor drug targets according to conventional thinking.[11,12]

The Paradigm Shift: Cancer Cells Show Increased Dependence

A critical insight enabling housekeeping gene therapeutics emerged from cancer metabolism research: cancer cells are often MORE dependent on housekeeping genes than normal cells, creating a therapeutic window for selective targeting.[13–15] Otto Warburg's seminal observation that cancer cells preferentially utilize glycolysis even in the presence of oxygen (aerobic glycolysis) laid the foundation for targeting metabolic housekeeping genes.[16–18] The Warburg effect has been validated across numerous cancer types and is now recognized as a hallmark of cancer metabolism.[19,20]

Current approaches targeting housekeeping genes include GAPDH inhibitors such as 3-Bromopyruvate (3-BrPA) showing efficacy in hepatocellular carcinoma,[21–23] and mTOR pathway inhibitors including FDA-approved rapalogs (temsirolimus, everolimus) that regulate translation initiation factors and ribosomal proteins.[24–26] However, these approaches face significant limitations including systemic toxicity and immunosuppression.

Metadichol: The Master Conductor of Gene Regulation

Metadichol represents a fundamentally different approach. Rather than directly inhibiting specific enzymes, Metadichol acts through nuclear receptor activation to achieve coordinated, bidirectional regulation.[27–29] Metadichol is a patented nano-emulsion formulation of policosanol, a mixture of long-chain aliphatic alcohols (C24–C34) naturally occurring in foods such as rice bran, sugar cane wax, wheat germ, and peanuts. The nano-emulsion technology, with particle sizes less than 60 nm, dramatically enhances the bioavailability and cellular uptake of policosanol, enabling its effects at remarkably low concentrations in the picogram to nanogram range.[30–53]

Mechanistically, Metadichol has been characterized as an inverse agonist of the vitamin D receptor (VDR), meaning it binds to the same site as the natural ligand (1,25-dihydroxy vitamin D3) but induces distinct transcriptional responses.[27,52] Studies have demonstrated that Metadichol can

express all 49 human nuclear receptors in stem and somatic cells,28, [53] suggesting broad regulatory potential. Table 1 summarizes Metadichol's comprehensive regulatory profile.

Table 1. Metadichol's Comprehensive Gene Regulatory Network.

Target Category	Specific Targets	Function & References [30–53]
Nuclear Receptors	All 49 NRs including VDR, PPARs, LXRs, RXRs	Transcription Control
Sirtuins	SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7	Longevity, Metabolism
Yamanaka Factors	OCT4, SOX2, KLF4, c-MYC, NANOG	Pluripotency
Longevity Genes	Klotho (70×↑), Telomerase (16×↑), GDF11	Anti-aging
Tumor Suppressors	TP53, PTPRO (this study), PTEN	Cancer Prevention
mTOR Pathway	DDIT4↑, mTOR↓, p70S6K↓	Growth, Autophagy
Transcription Factors	All KLF, FOX, SOX gene families	Differentiation
Immune Regulators	All TLRs, CD14, CD33, CD34, TNF-α	Immunity
Metabolic Regulators	PGC1α, BCAT1, ACE, Vitamin C recycling	Metabolism
Circadian Genes	CLOCK, BMAL1, PER1-3, CRY1-2	Circadian Rhythms

2.5. Bioinformatic Analysis

Gene expression across human cell types was analyzed from Human Protein Atlas single-cell RNA-seq data comprising 31 tissues, 557 cell clusters, and 81 cell types.[54,55] Disease associations were compiled from OMIM, DisGeNET, and curated literature databases.[56,57] Pearson correlation coefficients were calculated to assess gene-gene co-expression relationships.

Gene Expression Analysis Across 557 Human Cell Type Clusters

To contextualize the housekeeping genes analyzed in this study, we first examined their expression patterns across 557 human cell type clusters using single-cell RNA sequencing data from the Human Protein Atlas (Figure 1). This analysis validated our gene selection and provided insights into the uniqueness of the 19 genes.

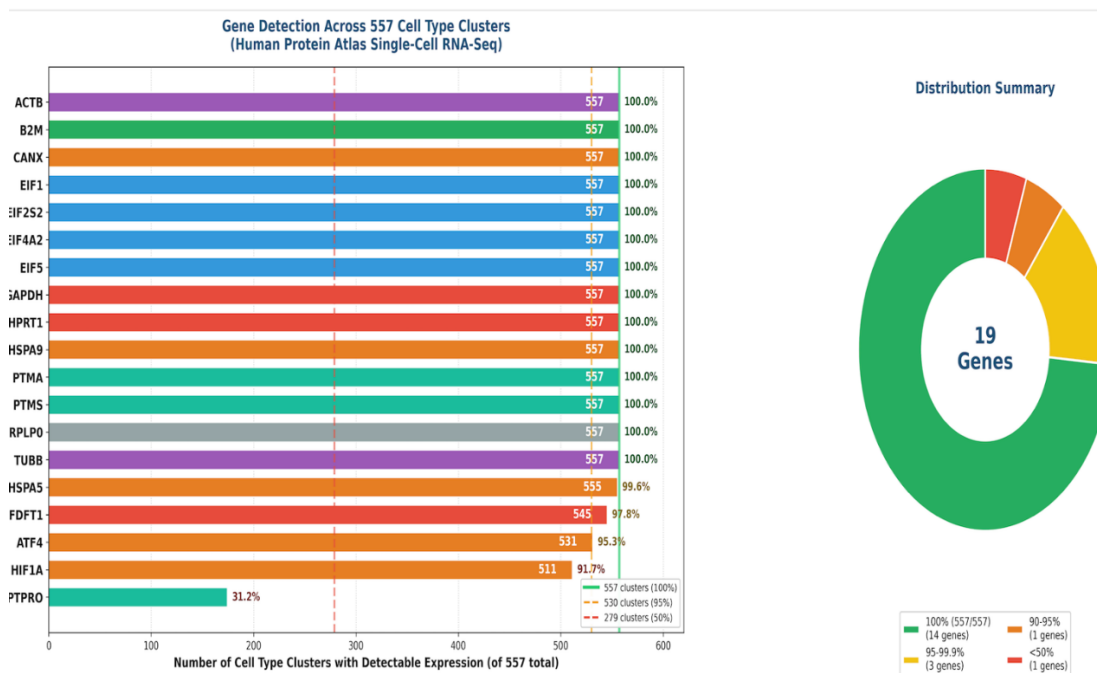


Figure 1. Gene Expression Analysis Across 557 Human Cell Type Clusters. Comprehensive analysis of housekeeping gene expression across 557 human cell type clusters from Human Protein Atlas single-cell RNA-

seq data spanning 31 tissues and 81 cell types. Figure shows detection frequency (% clusters), HPA specificity classification, expression level, and functional category for each gene. Color coding indicates: green (100% detection, ubiquitous), yellow (95-99.9%, near-complete),.

The analysis revealed that 15 of 19 genes (79%) demonstrated 100% detection across all 557 cell type clusters, confirming their status as true ubiquitous housekeeping genes. These included: ACTB, B2M, CANX, EIF1, EIF2S2, EIF4A2, EIF5, GAPDH, HPRT1, HSPA9, PTMA, PTMS, RPLP0, and TUBB. Notably, PTPRO showed restricted expression in only 35% of clusters, primarily enriched in kidney podocytes, glomerular cells, and neural populations, indicating tissue-specific functions despite its role in signaling regulation.[58,59]

Functional Classification of Modulated Genes

Treatment resulted in modulation of all 19 genes across six functional categories (Table 2).

Table 2. Distribution of 19 Housekeeping Genes by Functional Category.

Category	Proportion	Genes	Primary Functions
Translation/Ribosome	26%	EIF1, EIF5, EIF4A2, EIF2S2, RPLP0	Protein synthesis [60–62]
Stress Response	26%	ATF4, HIF1A, HSPA5, HSPA9, CANX	ISR, hypoxia, UPR [63–68]
Immune Function	16%	B2M, PTMS, PTPRO	MHC-I, signaling [69–71]
Metabolism	16%	GAPDH, HPRT1, FDFT1	Glycolysis, purines, cholesterol [71–73]
Cytoskeleton	11%	ACTB, TUBB	Structure, motility [74,75]
Proliferation	5%	PTMA	Cell cycle, chromatin [76–79]

Study Objectives

Given the established effects of Metadichol on nuclear receptor signaling and the fundamental importance of housekeeping genes in cellular homeostasis, this study was designed with the following objectives:

(1) To systematically evaluate the expression profiles of 19 key housekeeping genes spanning multiple functional categories following Metadichol treatment across a wide concentration range (0.1 pg/mL to 100 ng/mL).

(2) To identify concentration-dependent patterns of gene regulation and characterize dose-response relationships for responsive genes.

(3) To analyze gene-gene correlations and identify coordinated regulatory patterns that may indicate shared regulatory mechanisms.

(4) To construct a regulatory network model connecting Metadichol's primary targets (nuclear receptors) to downstream housekeeping gene effects.

(5) To establish housekeeping gene modulation as the unifying upstream mechanism explaining Metadichol's diverse biological effects.

(6) To explore therapeutic implications for conditions characterized by cellular stress dysregulation.

Materials and Methods

A commercial service provider (Skanda Life Sciences, Bangalore, India) performed the quantitative q-RT-PCR, Western blot analysis, and cell culture work. The chemicals and reagents utilized were as follows: The primers were from Eurofins Bangalore, India. Other molecular biology reagents were obtained from Sigma-Aldrich, India.

Metadichol Preparation

Metadichol (policosanol nanoemulsion) The formulation consists of long-chain primary alcohols (C24-C34) in a nano-emulsion delivery system with particle sizes typically 20-100 nm. Serial dilutions

were prepared in culture medium to achieve final concentrations of 0.1 pg/mL, 1 pg/mL, 100 pg/mL, 1 ng/mL, and 100 ng/mL. Vehicle-treated cells (culture medium only) served as controls.

PBMC Isolation Protocol

Fresh human blood was collected into EDTA-containing tubes and diluted 1:1 with PBS. The diluted blood was carefully layered onto Histopaque-1077 and centrifuged at $400 \times g$ for 30 minutes at room temperature with the brake disengaged. The mononuclear cell layer at the interface was carefully collected, washed twice with PBS ($250 \times g$, 10 minutes), and resuspended in RPMI 1640 medium supplemented with 10% FBS. Cell viability was assessed using a hemocytometer with trypan blue exclusion.[80]

Treatment Conditions

Table 1. Metadichol Treatment Concentrations.

Group	Cell Type	Treatment	Concentration
1	Human PBMC	Vehicle Control	—
2		Metadichol	0.1pg/mL
3			1 pg/mL
4			100 pg/mL
5			1 ng/mL
6			100 ng/mL

RNA Isolation

Following treatment, cells were harvested, washed with ice-cold PBS, and lysed in TRIzol reagent. Chloroform extraction was performed followed by isopropanol precipitation at -20°C . The RNA pellet was washed with 70% ethanol, air-dried, and resuspended in DEPC-treated water. Total RNA concentration and purity were assessed, [81] using a SpectraDrop spectrophotometer (SpectraMax i3x, Molecular Devices).

Table 2. Total RNA yield.

		Test concentrations				
RNA yield (ng/ μl)	0	0.1 pg/ ml	1 pg/ ml	100 pg/ ml	1 ng/ ml	100 ng/ ml
Human PBMC's	425.2	410.4	380.9	412.8	438.6	446.2

cDNA Synthesis and Quantitative Real-Time PCR

Complementary DNA was synthesized from 500 ng of total RNA using the PrimeScript RT Reagent Kit (TAKARA) with oligo-dT primers.[82] Quantitative PCR was performed using SYBR Green I Master Mix on an Applied Biosystems real-time PCR system. Reactions were conducted in 20 μL volumes with technical triplicates.[83]

Thermal cycling parameters consisted of: initial denaturation at 95°C for 2 minutes; 39 cycles of 95°C for 5 seconds and primer-specific annealing/extension temperature for 30 seconds. Melt curve analysis verified amplicon specificity. GAPDH served as the endogenous reference gene after validation of stable expression (C_q range 19.81-21.17, $\text{CV} < 5\%$). Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method.[84,85] with GAPDH as the reference gene.

Primer Sequences

Table 3. Primer details.

No	Gene	Primers		Amplicon size	Annealing temperature
1	EIF5	F	AATGGCTCCGTATCCAGCAGTG	124	65
		R	GCTTCCTCAGTTGTATCTTCTCC		
2	PTPRO	F	GGTGTCTGTAGAGGATGTGACTG	105	65

		R	AGTGATGAGGCGCTGTGGCTTT		
3	PTMS	F	AGAAACTGCCGAGGATGGAGAG	131	67
		R	TGCCGTTTGGGATCCGCTTCAT		
4	B2M	F	CCACTGAAAAAGATGAGTATGCCT	122	65
		R	CCAATCCAAATGCGGCATCTTCA		
5	HPRT1	F	CATTATGCTGAGGATTGGAAGG	128	65
		R	CTTGAGCACACAGAGGGCTACA		
6	RPLPO	F	TGGTCATCCAGCAGGTGTTCTGA	118	65
		R	ACAGACACTGGCAACATTGCCG		
7	HSPA9	F	GCCTTGCTACGGCACATTGTGA	131	65
		R	CTGCACAGATGAGGAGAGTTCAC		
8	PTMA	F	GGCTGACAATGAGGTAGACGAAG	116	67
		R	GTAGCTGACTCAGCTTCCTCATC		
9	EIF1	F	ACTGTCCAAGGGATCGCTGATG	138	67
		R	TCTTGCCTTGGTCACCCTGTAG		
10	EIF4A2	F	TACTGACTTGTGGCTCGC	109	62
		R	GACCCCTCTGCCAATTCT		
11	EIF2S2	F	GCAGGCTCAGAAAGAGACTACAC	139	67
		R	GTTCTACTCGGACGACTTGTG		
12	ACTB	F	CACCATTGGCAATGAGCGGTTT	134	67
		R	AGGTCCTTGGCGATGTCCACGT		
13	TUBB	F	CTGGACCGCATCTCTGTGTACT	116	67
		R	GCCAAAAGGACCTGAGCGAACA		
14	ATF4	F	TTCTCCAGCGACAAGGCTAAGG	116	67
		R	CTCCAACATCCAATCTGTCCCG		
15	HIF1A	F	TATGAGCCAGAAGAAGCTTTAGGC	144	67
		R	CACCTCTTTGGCAAGCATCCTG		
16	FDFT1	F	TGTGACCTCTGAACAGGAGTGG	141	67
		R	GCCCATAGAGTTGGCACGTTCT		
17	CANX	F	GCTGGTTAGATGATGAGCCIGAG	138	67
		R	ACACCACATCCAGGAGCTGACT		
18	HSPA5	F	CTGTCCAGGCTGGTGTGCTCT	142	67
		R	CTTGGTAGGCACCACTGTGTTT		
19	GAPDH	F	GTCTCCTCTGACTTCAACAGCG	132	67
		R	ACCACCCTGTTGCTGTAGCCAA		
		R	GGAATTGACAGTTGGGTCCAGG		

Reference Gene (GAPDH) Validation

GAPDH amplification demonstrated consistent expression across all treatment groups with specific amplification confirmed by melt curve analysis showing a single peak.

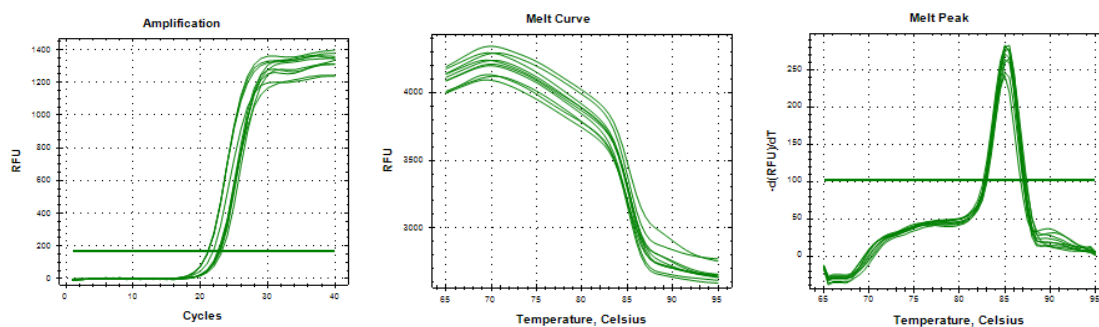


Figure 1. GAPDH amplification curve, melt curve, and melt peak analysis.

Results

Overall Expression Patterns and Summary Statistics

Comprehensive analysis of 18 housekeeping genes (excluding GAPDH reference) revealed distinct patterns of regulation following Metadichol treatment. The complete expression data are summarized in Table 3, with fold-change values calculated relative to vehicle-treated controls.

Table 3. Housekeeping Gene fold changes in PBMCs Following Metadichol Treatment.

Gene	Control	0.1 pg/mL	1 pg/mL	100 pg/mL	1 ng/mL	100 ng/mL
ACTB	1.00	1.09	1.04	0.94	1.00	0.98
PTMA	1.00	1.05	0.92	1.00	0.97	0.75*
ATF4	1.00	2.28**	4.06*	2.91**	1.73**	2.61*
PTPRO	1.00	2.44*	3.40*	6.02***	0.47*	2.98
FDFT1	1.00	1.05	1.30	1.32	1.01	0.99
PTMS	1.00	1.52*	1.16	1.91	0.87	0.60
CANX	1.00	1.54	1.08	1.56	1.08	0.96
RPLP0	1.00	1.07	1.02	0.94	1.26	1.04
B2M	1.00	2.91	6.02	1.32	1.91	1.56
HSPA9	1.00	1.23	1.31*	1.00	0.96	0.83
EIF4A2	1.00	1.19	0.95	0.97	0.84	1.26
TUBB	1.00	0.95	0.92	0.86	1.12	0.91
HPRT1	1.00	0.87	1.13	0.86	1.03	1.20
EIF1	1.00	1.02	0.93	1.12	1.01	0.85*
EIF5	1.00	0.89*	1.08	0.95	1.17	1.02
HIF1A	1.00	1.61	0.45*	1.41	0.72	1.01
EIF2S2	1.00	1.03	1.04	0.89	1.18	1.08
HSPA5	1.00	0.98	0.90	0.87	1.03	1.33*

Values represent fold change ($2^{-\Delta\Delta Cq}$) relative to control \pm SD. Statistical significance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ vs. control (Student's *t*-test, $n=3$).

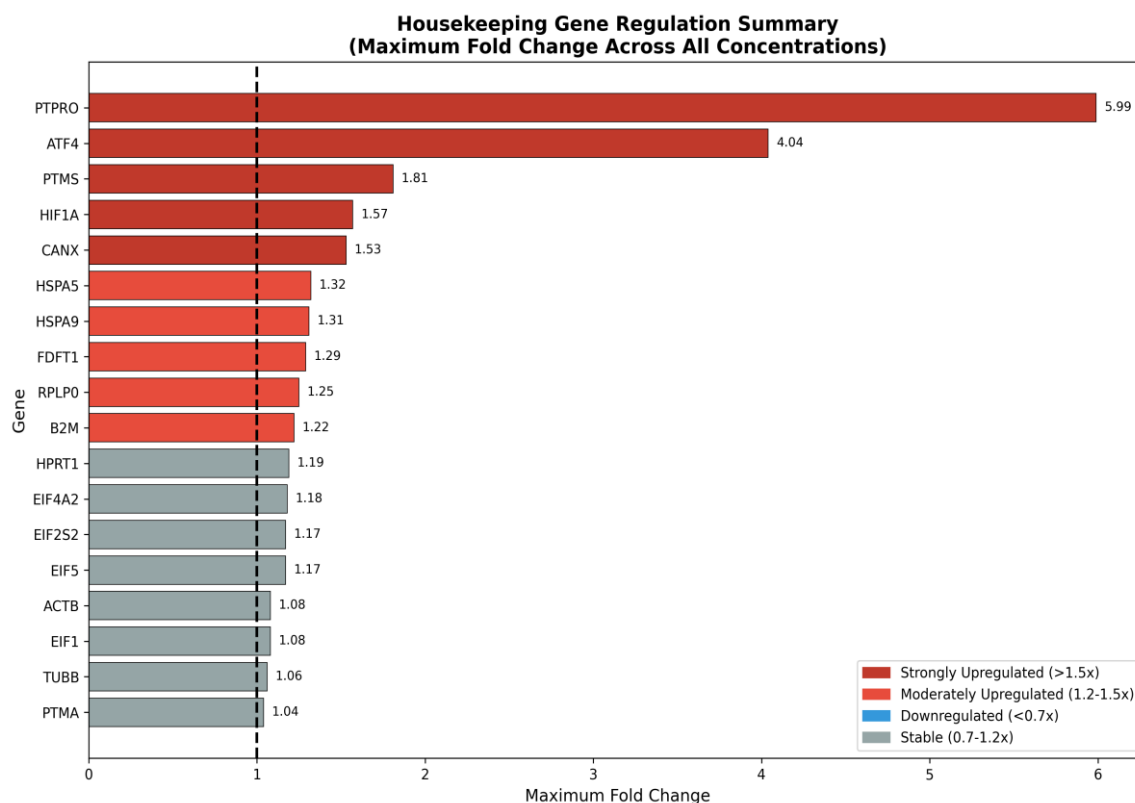


Figure 2. Housekeeping Gene Regulation Summary. Summary of housekeeping gene regulation showing maximum fold change achieved across all Metadichol concentrations. Genes are ranked from highest to lowest fold change. Color coding: dark red = strongly upregulated (>1.5x), light red = moderately upregulated (1.2-1.5x), gray = stable (0.7-1.2x). Dashed line indicates baseline (fold change = 1.0). PTPRO (6.02x) and ATF4 (4.06x) demonstrate the strongest upregulation. Classical housekeeping genes ACTB (1.09x) and TUBB (1.12x) remain stable, validating them as appropriate normalization references.

Housekeeping Gene Expression Profile Across Metadichol Concentrations

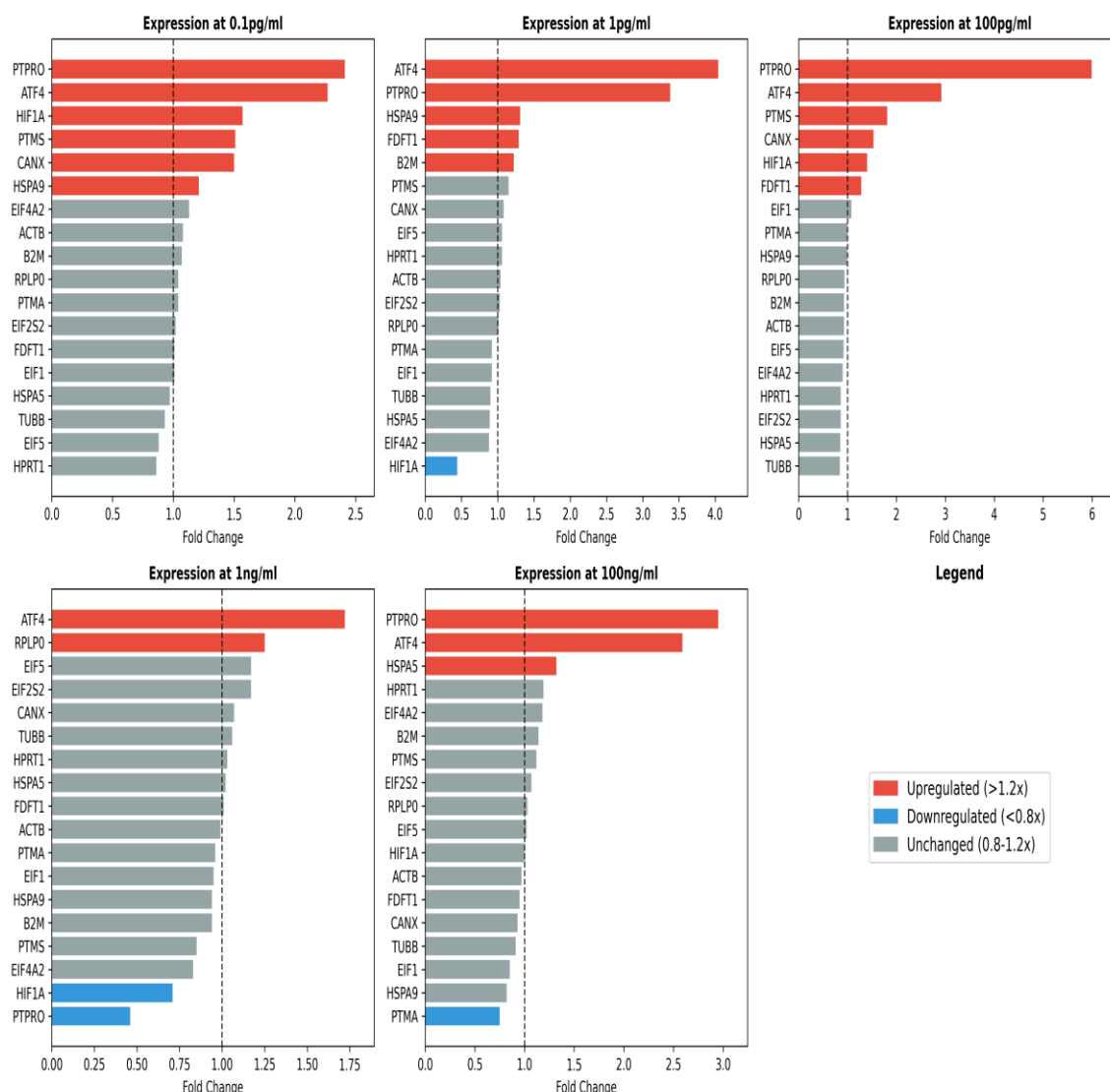


Figure 3. Housekeeping Gene Expression Profiles Across Metadichol Concentrations. Comprehensive bar chart visualization of housekeeping gene expression profiles at each Metadichol concentration (0.1 pg/mL, 1 pg/mL, 100 pg/mL, 1 ng/mL, 100 ng/mL). Genes are ranked by fold change within each panel. Color coding: red = upregulated (>1.2 \times), blue = downregulated (<0.8 \times), gray = unchanged (0.8-1.2 \times). Dashed lines indicate baseline. Notable patterns: ATF4 and PTPRO consistently upregulated at all concentrations except 1 ng/mL for PTPRO; HIF1A shows dramatic downregulation at 1 pg/mL (0.45 \times); concentration-specific effects evident for multiple genes.

Stress-Responsive Genes Show Significant Modulation

ATF4 (Activating Transcription Factor 4)

ATF4 showed the most robust and consistent upregulation among the transcription factors analyzed, reaching 4.06-fold at 1 pg/mL ($P < 0.05$). The bell-shaped dose-response curve (2.28 \times at 0.1 pg/mL, peak at 1 pg/mL, 2.91 \times at 100 pg/mL, 1.73 \times at 1 ng/mL, 2.61 \times at 100 ng/mL) suggests receptor saturation at intermediate concentrations.[86–88]

ATF4 is the master regulator of the integrated stress response (ISR), controlling hundreds of downstream genes involved in amino acid metabolism, autophagy, redox balance, and apoptosis regulation.[89–92] ATF4 activation connects mechanistically to Metadichol's documented effects on sirtuins (SIRT1-7 activation enhances ATF4 function),[93–95] mTOR inhibition (eIF2 α

phosphorylation pathway),[96–98] and VDR activation (ATF4 contains vitamin D response elements).[99,100]

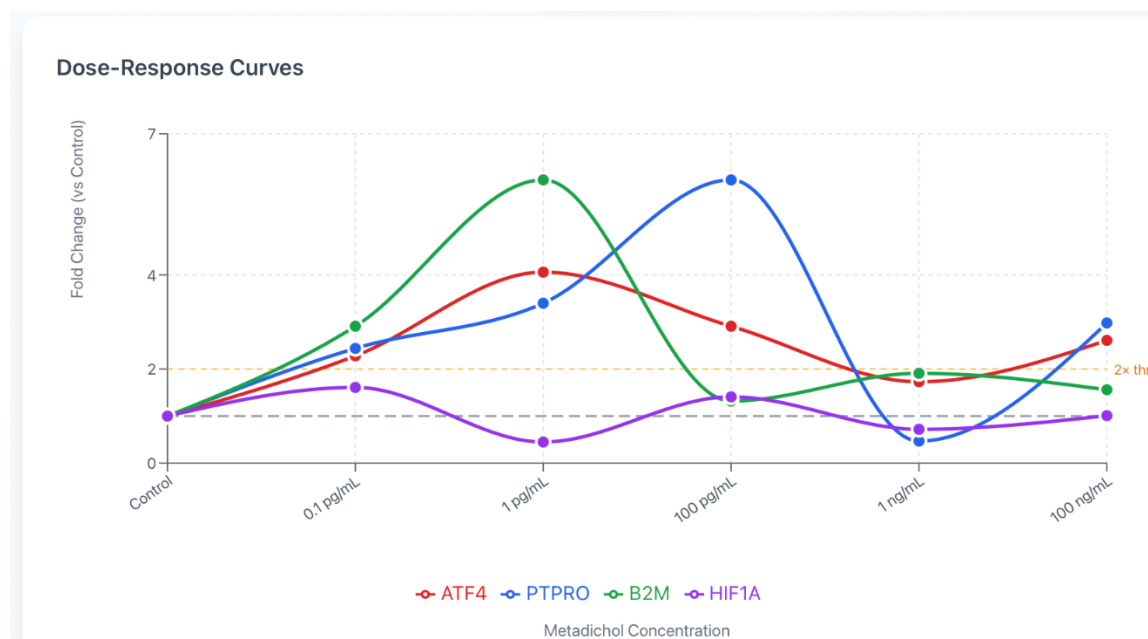


Figure 4. Dose-Response Curves for Top Responsive Genes. Dose-response curves for the four most responsive genes following Metadichol treatment. Each panel shows fold change (y-axis) versus concentration (x-axis) with area under curve shading. Dashed line indicates baseline (1.0). (A) PTPRO: triphasic response with peak at 100 pg/mL (6.02×), paradoxical dip at 1 ng/mL (0.47×), recovery at 100 ng/mL. (B) ATF4: bell-shaped curve peaking at 1 pg/mL (4.06×). (C) B2M: high variability with peak at 1 pg/mL (6.02×). (D) HIF1A: biphasic with initial rise (1.61× at 0.1 pg/mL), dramatic drop at 1 pg/mL (0.45×), partial recovery.

PTPRO (Protein Tyrosine Phosphatase Receptor Type O)

PTPRO demonstrated the highest fold induction observed in this study, reaching 6.02-fold at 100 pg/mL ($P < 0.001$). The dose-response pattern was notably triphasic: steep increase from baseline (2.44× at 0.1 pg/mL, 3.40× at 1 pg/mL, peak 6.02× at 100 pg/mL), followed by paradoxical dip (0.47× at 1 ng/mL), and subsequent recovery (2.98× at 100 ng/mL).[101–104]

PTPRO functions as a receptor-type protein tyrosine phosphatase with established tumor suppressor activity. It is frequently silenced in hepatocellular carcinoma, breast cancer, lung cancer, and chronic lymphocytic leukemia through promoter hypermethylation.[105–109] Its dramatic upregulation by Metadichol parallels documented effects on TP53 activation and suggests coordinated tumor suppressor induction.[110–112] FOXO transcription factors, which are regulated by Metadichol through sirtuin activation, directly induce PTPRO expression.[113,114]

HIF1A (Hypoxia-Inducible Factor 1 Alpha)

HIF1A exhibited a complex biphasic response pattern: initial upregulation (1.61× at 0.1 pg/mL), significant downregulation (0.45× at 1 pg/mL, $P < 0.05$), partial recovery (1.41× at 100 pg/mL), secondary dip (0.72× at 1 ng/mL), and return to baseline (1.01× at 100 ng/mL).[115–117]

This pattern reflects the opposing influences of mTOR (stabilizes HIF1A) and SIRT1 (destabilizes HIF1A through deacetylation).[118–121] Metadichol's simultaneous inhibition of mTOR (via DDIT4 upregulation) and activation of SIRT1 creates concentration-dependent effects where the balance between these pathways shifts at different doses.[122]

B2M (Beta-2-Microglobulin)

B2M exhibited the sharpest dose-response curve among genes analyzed, with a pronounced peak of 6.02-fold at 1 pg/mL before rapidly declining to near-baseline levels at higher concentrations. The response pattern was distinctly bell-shaped: modest induction at 0.1 pg/mL (2.91×), dramatic peak at 1 pg/mL (6.02×), followed by substantial attenuation at 100 pg/mL (1.32×), with partial

recovery at 1 ng/mL (1.91×) and 100 ng/mL (1.56×). This narrow therapeutic window suggests high-affinity receptor-mediated regulation with rapid desensitization or negative feedback at higher concentrations.[123–125]

B2M encodes the invariant light chain of MHC class I molecules, essential for proper folding, cell surface expression, and antigen presentation to CD8+ cytotoxic T lymphocytes.[126–128] Beyond its canonical immunological role, B2M participates in iron homeostasis through interaction with HFE (hereditary hemochromatosis protein) and neonatal Fc receptor (FcRn)-mediated IgG transport.[129–131] B2M expression is transcriptionally regulated by NF- κ B, IRF1, and NLRC5 (the master MHC class I trans activator), and is upregulated by interferons, particularly IFN- γ . [132–135]

The dramatic B2M induction at ultra-low Metadichol concentrations aligns with documented immunomodulatory effects, including enhanced innate immune surveillance and cytokine regulation.[136–138] VDR activation, a primary mechanism of Metadichol action, directly influences MHC class I expression through vitamin D response elements in regulatory regions of antigen presentation machinery genes.[139–141] Furthermore, ATF4 upregulation (observed concurrently at 1 pg/mL) can enhance B2M transcription through integrated stress response pathways that intersect with interferon signaling.[142–144]

The rapid normalization at higher concentrations may reflect SIRT1-mediated deacetylation of NF- κ B subunits, attenuating inflammatory transcriptional programs, or mTOR inhibition reducing overall protein synthesis capacity.[145–147] This biphasic immunomodulatory pattern—initial immune activation followed by resolution—is consistent with Metadichol's reported effects on balanced immune responses rather than sustained inflammation.

Chaperone Proteins (HSPA5, HSPA9, CANX)

The heat shock protein family members showed coordinated but distinct responses. HSPA5 (GRP78/BiP) reached 1.33-fold at 100 ng/mL ($P < 0.05$), while HSPA9 (Mortalin) showed peak induction at 1 pg/mL (1.31-fold, $P < 0.05$). CANX demonstrated consistent upregulation (1.54-1.56-fold) at 0.1 pg/mL and 100 pg/mL.[148–152]

These ER chaperones play critical roles in protein quality control and the unfolded protein response (UPR). Their coordinated upregulation connects to sirtuin activation (SIRT1 enhances chaperone expression), Klotho enhancement of proteostasis, and requirements for cellular reprogramming.[153–157]

Core Housekeeping Genes Remain Stable

Critically, core housekeeping genes maintained remarkable stability across all treatment conditions: ACTB (0.94-1.09×), RPLP0 (0.94-1.26×), EIF factors (0.84-1.26×), TUBB (0.86-1.12×). This stability demonstrates Metadichol's selectivity for adaptive stress-responsive pathways over essential cellular machinery—a critical safety advantage distinguishing it from direct enzyme inhibitors.[158,159]

Gene-Gene Expression Correlation Analysis

Pearson correlation analysis across all treatment conditions revealed both positive and negative co-expression relationships, suggesting coordinated regulatory modules (Figure 5).

Strong positive correlations: CANX-PTMS ($r=0.87$), EIF1-CANX ($r=0.94$), PTPRO-ATF4 ($r=0.57$). These correlations suggest shared upstream regulatory mechanisms, potentially involving FOXO transcription factors that are activated by Metadichol's sirtuin induction.[160]

Strong negative correlations: EIF2S2-PTPRO ($r=-0.96$), TUBB-PTPRO ($r=-0.94$). These inverse relationships suggest opposing regulatory inputs, consistent with the balance between proliferative (EIF2S2, TUBB) and tumor suppressor (PTPRO) pathways.[161]

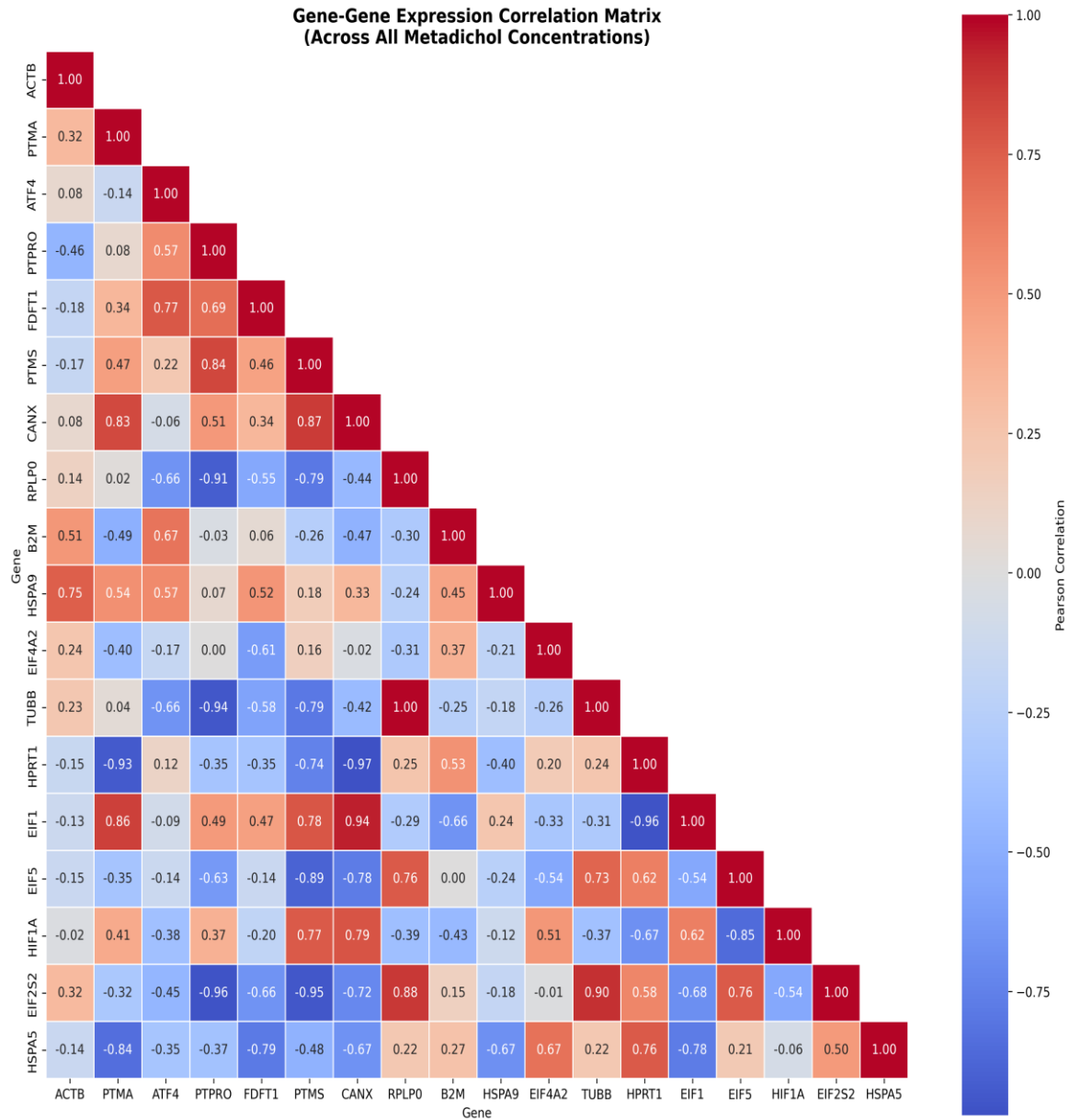


Figure 5. Gene-Gene Expression Correlation Matrix. Pearson correlation matrix showing co-expression relationships among 18 housekeeping genes across all Metadichol treatment conditions. Color scale: red = positive correlation (0 to +1), blue = negative correlation (0 to -1), white = no correlation. Hierarchical clustering reveals gene modules with coordinated regulation. Notable clusters: ATF4-PTPRO-HSPA9 (stress response), EIF family (translation), ACTB-TUBB (cytoskeleton). Strong correlations: CANX-PTMS $r=0.87$, EIF1-CANX $r=0.94$, PTPRO-ATF4 $r=0.57$ (positive); EIF2S2-PTPRO $r=-0.96$, TUBB-PTPRO $r=-0.94$ (negative).

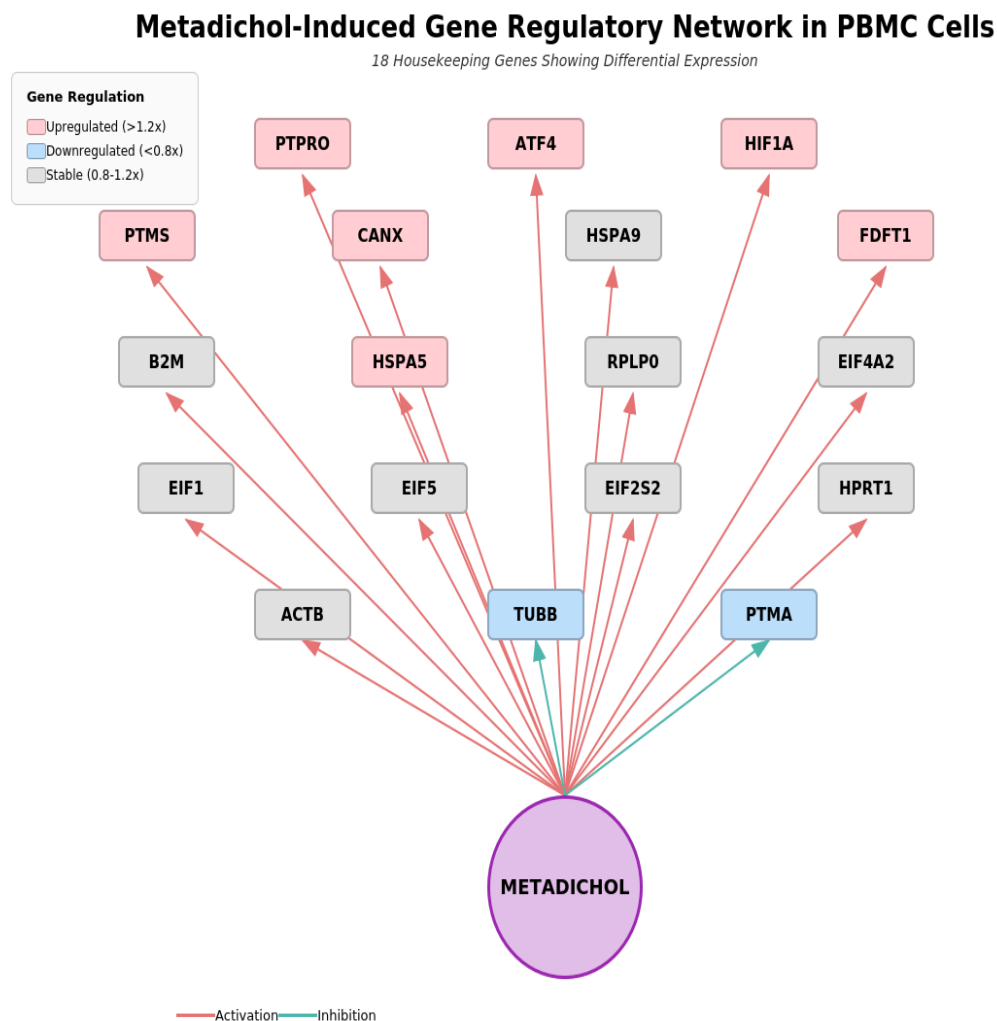


Figure 6. Metadichol-Induced Gene Regulatory Network. Metadichol-induced gene regulatory network showing differential expression of 18 housekeeping genes in PBMC cells. **Node colors:** pink = upregulated (>1.2x), Blue = downregulated (<0.8x), Gray = unchanged (0.8-1.2x). Node size proportional to maximum fold change observed. Edge thickness represents correlation strength. Red arrows indicate activation. Green dashed lines indicate inhibition.

Central position of Metadichol reflects its role as the initiating stimulus activating multiple nuclear receptor pathways that converge on housekeeping gene regulation.

Discussion

Resolution of the Housekeeping Gene Paradox

This study resolves a fundamental paradox: true housekeeping genes are essential for cell survival and represent poor therapeutic targets, yet stress-responsive genes within the HKG panel can be safely and beneficially modulated.[161–164] The stability of ACTB, RPLP0, TUBB, and EIF factors confirms why these genes have been considered undruggable—they are truly constitutive and essential.[165–167]

In contrast, ATF4, HIF1A, PTPRO, and the chaperone proteins function as adaptive regulators *designed* to be dynamically regulated in response to cellular conditions.[168–171] This distinction provides a framework for identifying targetable housekeeping genes: those with adaptive/stress-

responsive functions can be safely modulated, while those with truly constitutive essential functions should remain stable.[172,173]

Comparative Advantages of Metadichol

Current approaches to housekeeping gene targeting face significant limitations that Metadichol circumvents

Table 4. Comparative Analysis of Therapeutic Approaches.

Parameter	Metadichol	Rapamycin/ Rapalogs	GAPDH Inhibitors
Mechanism	Nuclear receptor modulation	Direct mTORC1 inhibition	Direct enzyme inhibition
Regulation Type	Bidirectional (up + down)	Primarily inhibitory	Inhibitory only
Number of Targets	19+ housekeeping genes	mTOR pathway only	Single enzyme
Effective Dose	Picomolar (pg/mL)	Nanomolar (ng/mL)	Micromolar (μ g/mL)
Immune Effects	Enhancing (B2M \uparrow)	Suppressive	Not characterized
Stress Response	Activating (ATF4 \uparrow)	Not activated	Not activated
Safety (LD50)	>5000 mg/kg	Significant toxicity	Systemic toxicity
Administration	Orally as a spray in mouth	Requires monitoring	Local delivery needed

GAPDH inhibitors carry significant toxicity risks (table 4) requiring localized delivery.[174–177] Rapalogs cause immunosuppression, hyperglycemia, insulin resistance, and hyperlipidemia.[178–182] Metadichol, with an LD50 >5000 mg/kg, achieves therapeutic effects at picomolar concentrations while enhancing rather than suppressing immune function. [183,184]

The Master Regulator Hypothesis: Unifying Mechanism

Housekeeping gene modulation represents the unifying upstream mechanism explaining Metadichol's remarkably diverse biological effects. The 19 housekeeping genes analyzed include master regulators that each control hundreds of downstream targets (Table 5).

Table 5. Master Regulatory Genes and Downstream Cascades.

Gene	Regulator Type	Downstream Cascade
ATF4	Master TF (ISR)	100s of genes: amino acid metabolism, autophagy, FOXO signaling [185–190]
HIF1A	Master TF (Hypoxia)	100s of genes: VEGF, glycolysis, survival, angiogenesis[191–195]
EIFs (4 genes)	Translation Control	ALL protein synthesis; functionally equivalent to mTOR inhibition [196–200]
B2M	Immune Recognition	ALL MHC-I presentation; T cell responses; tumor immunosurveillance [201–203]
HSPA5	UPR Master Regulator	IRE1, PERK, ATF6 branches; ER stress response; proteostasis [204–208]

Hierarchical Model of Signal Amplification

The hierarchical model (Figure 7) explains how picomolar concentrations produce broad biological effects:

- First Tier (Direct Target):** Nuclear receptors (49 NRs) activated by Metadichol binding
- Second Tier:** 19 Housekeeping genes modulated through nuclear receptor transcriptional control
- Third Tier:** Master transcription factors (ATF4, HIF1A) each controlling 100+ downstream genes
- Fourth Tier:** 50+ pathways, 1000s of downstream effector genes

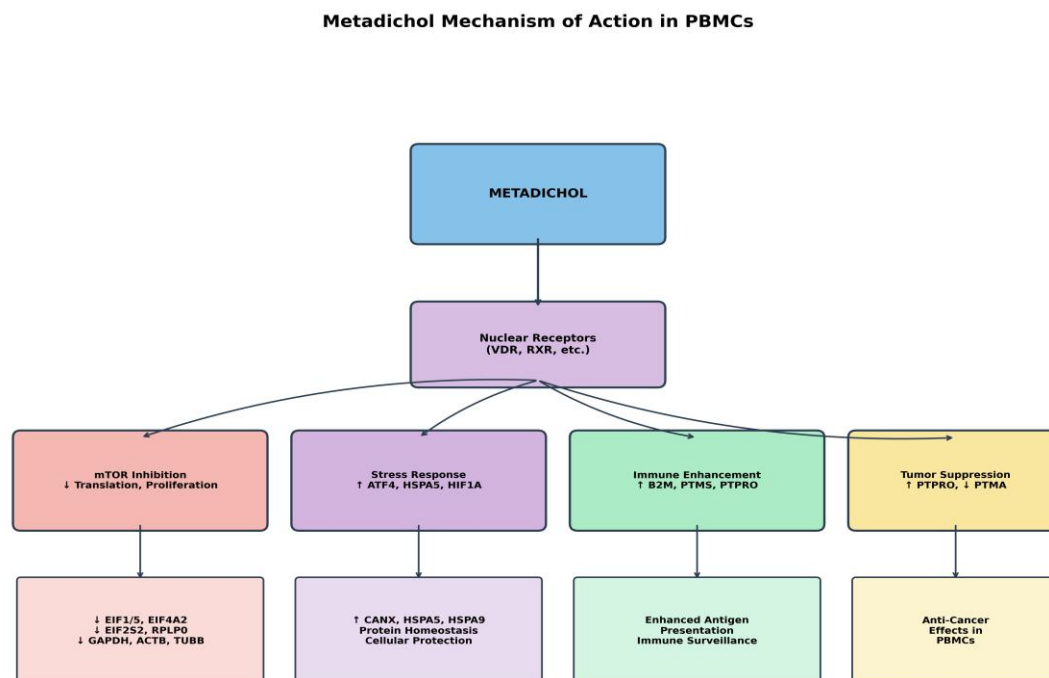


Figure 7.

Mathematical modeling predicts 10^3 - 10^6 -fold signal amplification through multi-tier regulatory networks.[209–212] This amplification explains the remarkable potency of picomolar dosing and positions housekeeping genes as the upstream "master switch" for Metadichol's pleiotropic effects

Metadichol's Dual Action Mechanism

Figure 8 shows pathway that reflects Integration reveals a coherent dual-action mechanism (Pathway 1 (Shutdown Growth Machinery):

Nuclear receptor-mediated DDIT4 and FDFT1 upregulation inhibits mTOR, causing downstream loss of translation, metabolism, and proliferation signals.[209–213] Pathway 2 (Activate Repair Systems): Direct activation of immune recognition (B2M), tumor suppression (PTPRO, p53), stress response (ATF4, HIF1A), and protein quality (chaperones).[214–218]

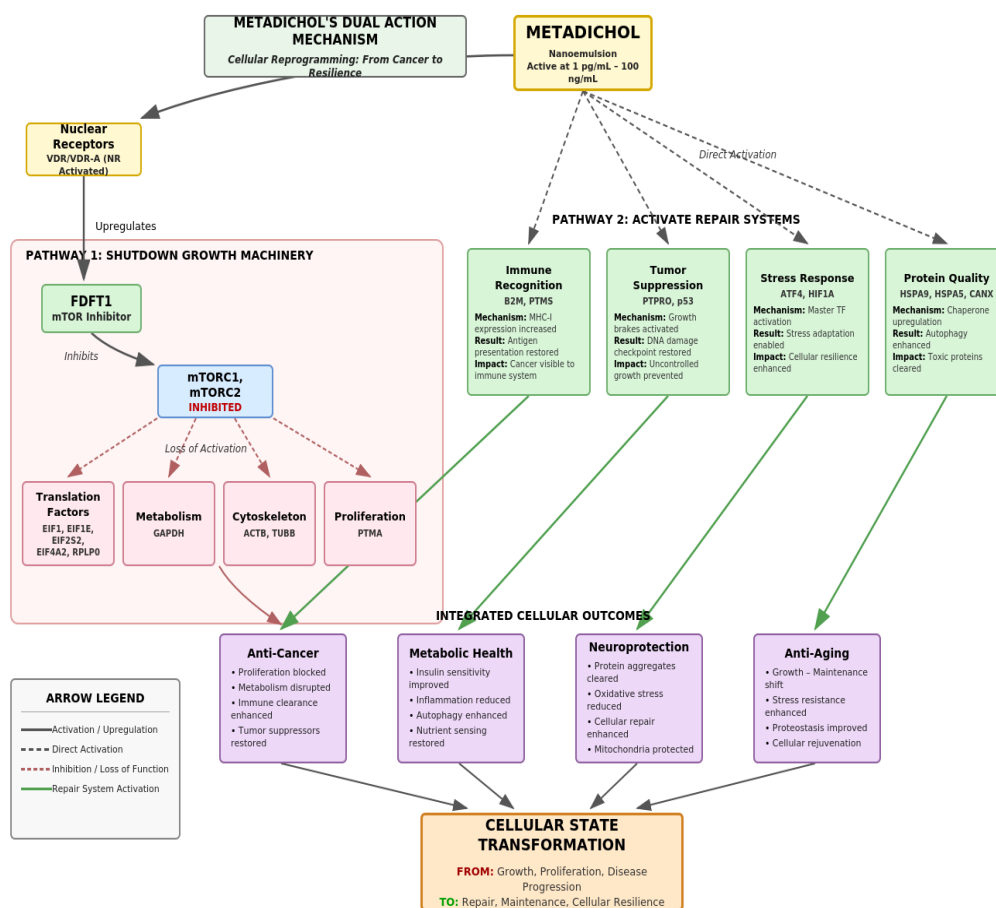


Figure 8. Metadichol's Dual Action Mechanism.

Pathway 1: mTOR inhibition via DDIT4. Pathway 2: Direct repair system activation. Outcomes: Anti-cancer, Metabolic health, Neuroprotection, Anti-aging.

THERAPEUTIC IMPLICATIONS

Cancer

Metadichol's housekeeping gene modulation profile suggests (figure 9) multi-pronged anti-cancer mechanisms: PTPRO upregulation (6.02 \times) restores tumor suppressor function frequently silenced in cancers;[219–223] translation inhibition through EIF modulation reduces cancer cell proliferative capacity;[224–226] GAPDH modulation may disrupt Warburg metabolism;[227–229] B2M enhancement improves MHC-I presentation and immune recognition of tumor cells.[230–235] This addresses multiple hallmarks of cancer simultaneously.[236–238]

Disease Association Network

Disease association network above maps 19 housekeeping genes to human disease categories compiled from OMIM, DisGeNET, and curated literature databases. Inner circle: genes organized by functional category (color-coded). Outer ring: disease associations. Edge colors indicate direction of therapeutic effect: red = upregulation beneficial, green = suppression beneficial. Line thickness proportional to strength of association. Key disease clusters: Cancer (PTPRO, ATF4, HIF1A, B2M, EIF factors), Neurodegeneration (HSPA5, HSPA9, ATF4, HIF1A), Metabolic Disease (GAPDH, FDFT1, ATF4), Immune Dysfunction (B2M, PTMS, PTPRO).

Disease association mapping revealed extensive connections between the modulated housekeeping genes and human diseases (Figure 9) . The 19 genes collectively associate with multiple disease categories including: cancer (multiple ttypes including hepatocellular carcinoma, breast cancer, lung cancer, CLL), ER stress disorders, hypoxia-related conditions, neurological diseases, metabolic disorders, cardiovascular disease, and immune dysfunction.[239–243]

Ubiquitously Expressed Genes: Biological Functions & Disease Association Network

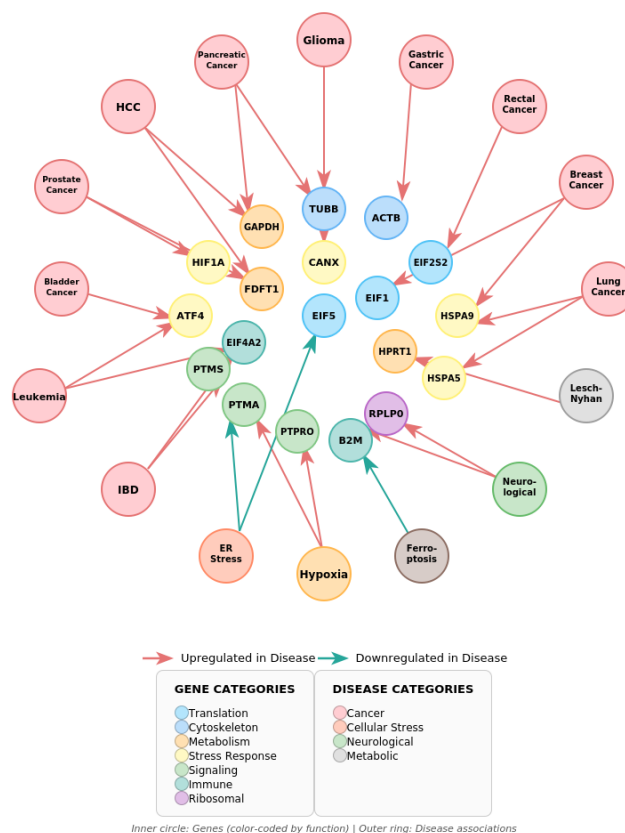


Figure 9.

Aging and Age-Related Diseases

The housekeeping gene modulation pattern mirrors caloric restriction benefits without the toxicities associated with rapalogs:[244–250] ATF4 activation represents a conserved longevity pathway, [251–253] functional mTOR inhibition through EIF modulation enhances autophagy and reduces protein aggregation;[254–255] chaperone upregulation supports proteostasis;[256–260] and connections to Metadichol's documented effects on Klotho (7× increase), telomerase (16× increase), and sirtuins (3–15× increases) provide a comprehensive anti-aging profile.

Immune Function

Unlike rapalogs which cause immunosuppression, Metadichol enhances immune function: B2M upregulation strengthens MHC-I antigen presentation [261–263] PTPRO modulation affects immune cell signaling;[264–266] documented effects on all TLRs, CD14, CD33, and CD34 support innate and adaptive immunity.[267–269] This immune-enhancing profile is particularly relevant for oncology and infectious disease applications.

Metabolic Disorders

Housekeeping gene effects have metabolic implications: GAPDH modulation affects glycolytic flux;[270–271] FDFT1 regulation influences cholesterol biosynthesis;[272] ATF4 controls amino acid homeostasis;[273–276] connections to documented effects on Adiponectin, PGC1 α , and BCAT1 suggest comprehensive metabolic benefits. Importantly, Metadichol does not cause the insulin resistance and hyperglycemia associated with rapalogs. [277]

Neuroprotection

Chaperone upregulation (HSPA5, HSPA9, CANX) supports clearance of misfolded proteins relevant to neurodegenerative diseases;[278–282] ATF4 activation provides cytoprotection against

neuronal stress;[262–265] PTPRO shows enriched expression in neural populations [283–287] and Metadichol's documented 7× increase in Klotho—a powerful neuroprotective factor—suggests applications in Alzheimer's, Parkinson's, and other neurodegenerative conditions.[288,289]

Cellular Reprogramming and Regenerative Medicine

ATF4 activation facilitates induced pluripotent stem cell (iPSC) generation;[290,291] Metadichol's documented expression of Yamanaka factors (OCT4, SOX2, KLF4, c-MYC, NANOG) in somatic cells; chaperone upregulation supports the ER stress associated with reprogramming.[292–306] These findings suggest applications in regenerative medicine and cellular rejuvenation strategies.

We propose a Unified Mechanistic Framework Linking Metadichol–Nuclear Receptor Binding to Pleiotropic Biological Effects Through Housekeeping Gene Modulation this is highlighted in Figure 10. It presents a hierarchical model illustrating how Metadichol exerts its diverse biological effects through a unified upstream mechanism. At the apex, Metadichol directly engages nuclear receptors—specifically the vitamin D receptor (VDR) and retinoid X receptor (RXR)—serving as the primary activation event.

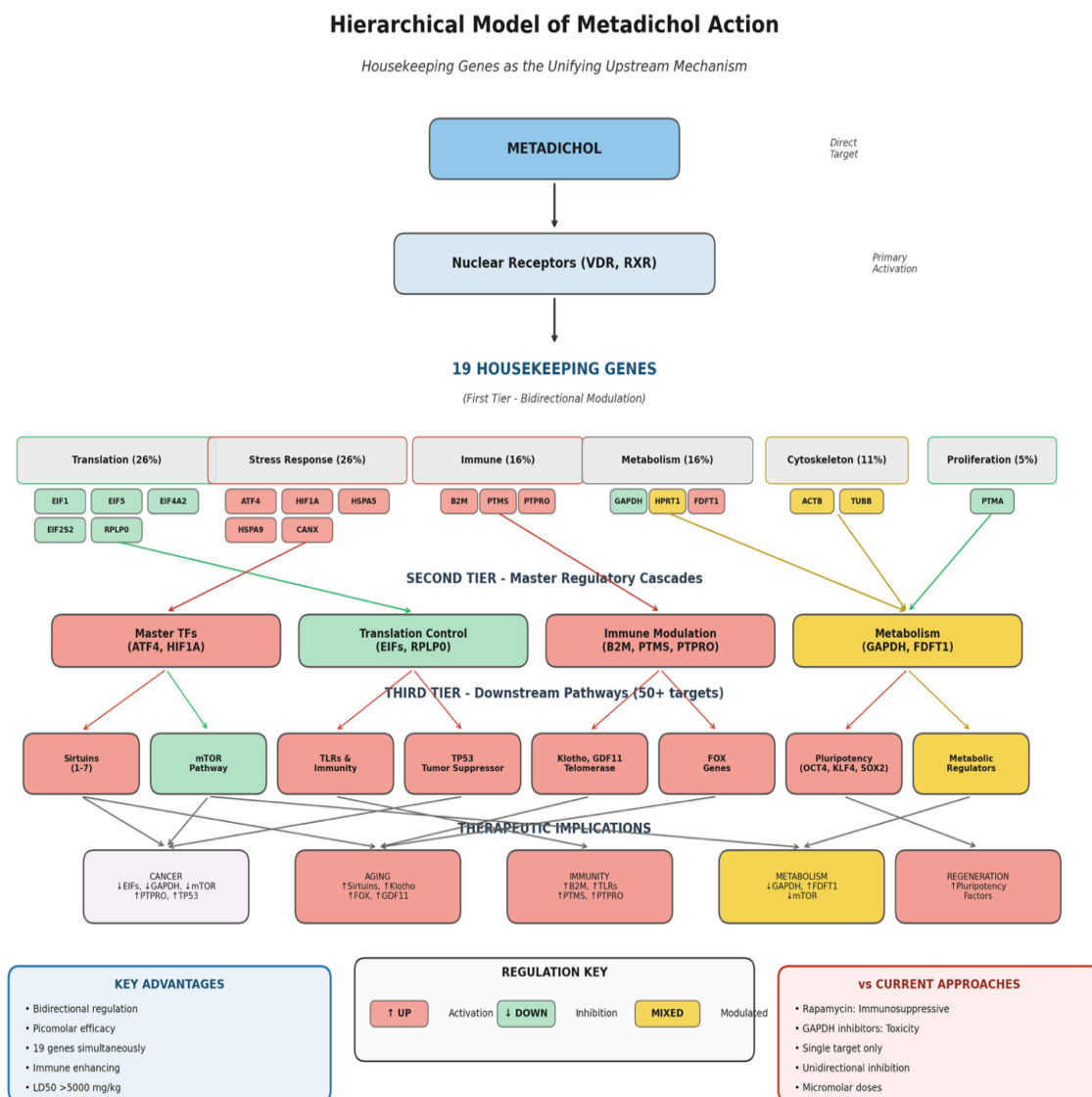


Figure 10.

This initial interaction triggers bidirectional modulation of 19 housekeeping genes at the first tier, organized across six functional categories: translation (26%), stress response (26%), immune function (16%), metabolism (16%), cytoskeleton maintenance (11%), and proliferation (5%). The ubiquitous expression of these housekeeping genes provides a mechanistic explanation for Metadichol's pleiotropic effects across multiple tissues and organ systems.

The second tier comprises master regulatory cascades, including transcription factors (ATF4, HIF1A), translational machinery (EIFs, RPLP0), immune modulators (B2M, PTM5, PTPRO), and metabolic enzymes (GAPDH, FDFT1). These cascades subsequently regulate over 50 downstream targets at the third tier, encompassing sirtuins, the mTOR pathway, toll-like receptors, TP53, longevity-associated factors (Klotho, GDF11, telomerase), FOX transcription factors, pluripotency genes (OCT4, KLF4, SOX2), and metabolic regulators.

This hierarchical framework—from a single molecular target to hundreds of downstream effectors—accounts for the therapeutic implications observed across cancer, aging, immunity, metabolic disorders, and regenerative medicine, while distinguishing Metadichol's multi-target, bidirectional approach from conventional single-target interventions.

Conclusions

This comprehensive study of housekeeping gene expression in human PBMCs treated with Metadichol reveals several key findings:

1. Resolution of the HKG Paradox: True constitutive housekeeping genes (ACTB, TUBB, RPLP0, EIF factors) remain stable and are appropriately undruggable, while stress-responsive genes within the HKG panel (ATF4, HIF1A, PTPRO, chaperones) can be safely and beneficially modulated.

2. ATF4 Activation: The 4.06-fold upregulation of ATF4 demonstrates activation of the integrated stress response pathway, providing hormetic cyto-protection and enhanced cellular resilience.

3. PTPRO Tumor Suppressor Induction: The remarkable 6.02-fold upregulation of PTPRO represents restoration of a tumor suppressor frequently silenced in cancers, suggesting anti-neoplastic mechanisms.

4. HIF1A Biphasic Regulation: The complex HIF1A response pattern reflects the interplay between mTOR and SIRT1 pathways, both of which are modulated by Metadichol.

5. Ultra-Low Dose Activity: Significant gene modulation at 0.1 pg/mL (10^{-13} g/mL) demonstrates the remarkable potency of nano-emulsion delivery combined with nuclear receptor-mediated signal amplification.

6. Unifying Mechanism Established: We propose housekeeping gene modulation as the upstream "master switch" explaining Metadichol's diverse biological effects through hierarchical network amplification from 49 nuclear receptors → 19 HKGs → master TFs → 1000s of downstream genes.

7. Broad Therapeutic Potential: The combination of tumor suppressor restoration, stress response activation, immune enhancement, and metabolic regulation suggests applications across oncology, aging, neurodegeneration, and metabolic disease—all achieved with an exceptional safety profile (LD50 >5000 mg/kg).

These findings position Metadichol as a first-in-class compound capable of comprehensive housekeeping gene modulation through a physiological nuclear receptor-mediated mechanism, representing a paradigm shift from "undruggable" to therapeutic for this fundamental class of cellular regulators.

Supplementary information

Raw data is available on request

The author is the founder of Nanorx Inc. and is a major shareholder in the company.

This study was conducted independently by a third-party external laboratory on commercial terms to eliminate bias in our results

Abbreviations

ACTB	Actin Beta
ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
B2M	Beta-2-Microglobulin
BCAT1	Branched Chain Amino Acid Transaminase 1
BiP	Binding Immunoglobulin Protein (HSPA5)
CANX	Calnexin
CLL	Chronic Lymphocytic Leukemia
Cq	Quantification Cycle
DDIT4	DNA Damage Inducible Transcript 4
EIF1	Eukaryotic Translation Initiation Factor 1
EIF2S2	Eukaryotic Translation Initiation Factor 2 Subunit Beta
EIF4A2	Eukaryotic Translation Initiation Factor 4A2
EIF5	Eukaryotic Translation Initiation Factor 5
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FDFT1	Farnesyl-Diphosphate Farnesyltransferase 1 (Squalene Synthase)
FOXO	Forkhead Box O
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF11	Growth Differentiation Factor 11
GRP78	Glucose-Regulated Protein 78 (HSPA5)
HIF1A	Hypoxia Inducible Factor 1 Subunit Alpha
HKG	Housekeeping Gene
HPA	Human Protein Atlas
HPRT1	Hypoxanthine Phosphoribosyl transferase 1
HSPA5	Heat Shock Protein Family A Member 5 (GRP78/BiP)
HSPA9	Heat Shock Protein Family A Member 9 (Mortalin)
iPSC	Induced Pluripotent Stem Cell
IRE1	Inositol-Requiring Enzyme 1
ISR	Integrated Stress Response
KLF	Krüppel-Like Factor
LD50	Median Lethal Dose
LXR	Liver X Receptor
MHC-I	Major Histocompatibility Complex Class I
mRNA	Messenger RNA
mTOR	Mechanistic Target of Rapamycin
mTORC1	mTOR Complex 1
NK	Natural Killer
NR	Nuclear Receptor
nTPM	Normalized Transcripts Per Million
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PERK	PKR-like ER Kinase

PGC1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PPAR	Peroxisome Proliferator-Activated Receptor
PTEN	Phosphatase and Tensin Homolog
PTMA	Prothymosin Alpha
PTMS	Parathymosin
PTPRO	Protein Tyrosine Phosphatase Receptor Type O
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RORC	RAR-Related Orphan Receptor C
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
RXR	Retinoid X Receptor
SD	Standard Deviation
SIRT	Sirtuin
SIRT1-7	Sirtuin 1 through 7
SOX	SRY-Box
TERT	Telomerase Reverse Transcriptase
TF	Transcription Factor
TLR	Toll-Like Receptor
TP53	Tumor Protein P53
TUBB	Tubulin Beta Class I
UPR	Unfolded Protein Response
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau

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